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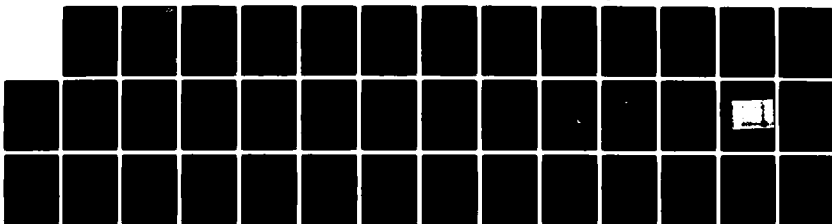
PURIFICATION OF N-ACETYL GALACTOSAMINIDASE BY
ISOELECTRIC FOCUSING(U) WAYNE STATE UNIV DETROIT MI
DEPT OF BIOCHEMISTRY R K BROWN 02 APR 83
N00014-78-C-0767

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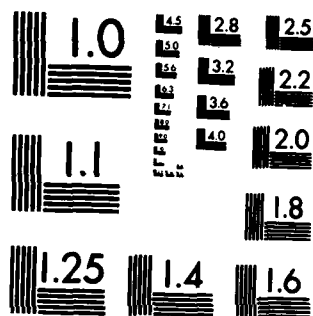
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Office of Naval Research
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Task No. NR 207-143

PURIFICATION OF N-ACETYL GALACTOSAMINIDASE

BY ISOELECTRIC FOCUSING

2 April 1983

BY

Ray K. Brown

Wayne State Univeristy

Department of Biochemistry

540 E. Canfield

Detroit, Michigan 48201

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This is the final technical report on Contract N00014-78-C-0767 NR 207-143. This project was directed at obtaining and purifying enzymes which could convert type A human red blood cells to type O. The latter can be given to all recipients and the availability of such cells would simplify inventory and cross matching during emergencies. Chemically the A antigen differs from the O antigen only in that it has an additional terminal sugar residue, an α linked N acetylgalactosamine. Two enzymes have been studied which remove this residue and convert A substance to O substance. One of these Azymes, α -N-acetylgalactosaminidase, was from *Clostridium perfringens* and was studied in collaboration with Dr. David Aminoff at the University of Michigan. The chief emphasis of the work with Clostridial Azyme was its purification by isoelectric focusing. Any bacterial enzyme contaminating red cells after their conversion from A to O could cause serious immunologic reactions in recipients. Human enzymes were studied in attempts to avoid this potential problem. The human Azyme was prepared from placenta. The studies with placental Azyme concerned its purification, properties and development of an assay for its activity toward type A red blood cells. Although preliminary experiments have been done with Azyme treated type A red cells high background and variability make their interpretation uncertain. Monoclonal antibodies of higher specificity should improve the assay. The investigator appreciates the support from the Office of Naval Research.

I. Literature Review

A few key references concerning the nature of type A determinants and of the experiments of others with enzymes which remove the terminal sugar from A substance are summarized in this section of the report. It is hoped these will provide background for the project. Readers familiar with the subject or interested only in the work done under the contract may wish to turn to page immediately.

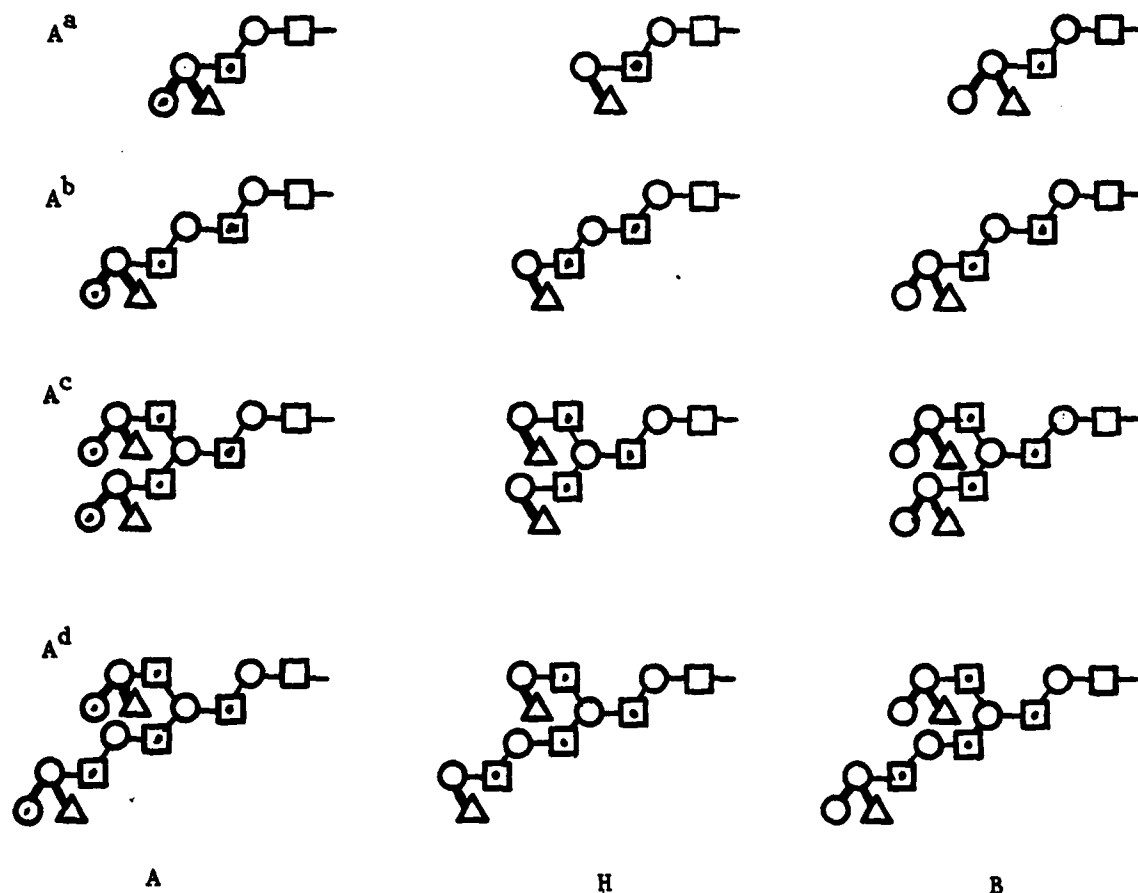
A. CHEMICAL NATURE OF BLOOD GROUP A DETERMINANTS

Substances which react with antibodies to the type A determinant of red blood cells occur in both the glycolipid and glycoprotein portions of the erythrocyte and are also widely distributed in many tissues. In all cases the antigenic component is the carbohydrate. The blood group A determinant differs from blood group O (called H by most investigators) in that a terminal α linked N-acetylgalactosamine has been added to the H group carbohydrate chain. Blood group B has an α linked galactose added to the terminus of the H chain i.e., α -N-acetylgalactosamine-H determinant=A determinants and α galactose-H determinant=B determinant. Removal of the terminal sugar from A or B determinants would produce O (i.e., H) determinants. Individuals, except those with blood type AB, normally have circulating antibodies to type A and/or type B blood groups. Type A individuals have anti B. Those with type B blood have anti A and type O individuals have antibodies against both A and B determinants. Type O cells react with neither A nor B antibodies and are universal donor cells. Conversion of A and B cells to type O would allow their administration to all recipients and simplify inventory of blood and speed up administration to recipients. This project has been directed at obtaining enzymes which convert A and B blood groups to type O.

Glycolipids with blood group activity

The glycolipids which contain blood group activity have been characterized recently. These studies are reviewed in depth by Hakomori (Seminars in Hematology 18, 39-61 (1981)). The carbohydrate moieties associated with blood group specificity are linked to ceramide

$\text{CH}_3(\text{CH}_2)_{12}\text{CH} \begin{matrix} \text{OH} \\ | \\ \text{NH-COR} \end{matrix} \text{CH-CH-CH}_2 \text{OH}$ through the terminal OH. There is considerable heterogeneity of blood group A, B, H (O) determinants. In all cases however the A determinants differ from the H determinants by addition of an α linked N-acetylgalactosamine and the B determinants by an α -galactose. The following structures illustrate these principles. In these schematic structures galactosyl = O, N-acetylgalactosaminyl = Θ , glucosyl = \square , N-acetylglucosaminyl = \blacksquare , fucosyl = Δ . β linkages are indicated by light bonds and α linkages by heavy bonds. The right most residue is linked to ceramide. Each linkage is from the 1 carbon of the left sugar residue to the 6 (\searrow), 4 ($-$), 3 (\nearrow), or 2 (\hookrightarrow) carbon of the sugar on the right.



In each case the H determinant is identical with the A or B determinant except for the terminal sugar (left most N acetylgalactosamine(s) (Θ) for A and galactose (O) for B). A^a , A^b and A^c have been converted to H_1 , H_2 and H_3 by hog liver Azyme (Hakomori, S. and Watanabe, K. in Glycolipid Methodology, L.A. Witting, Ed., American Oil Chemists Society, 1976, pgs. 13-47; see page 32). The structure of A^d was determined by Fukuda and Hakomori (J. Biol.

Chem. 257, 446-455 (1982)). The sub types differ branching or by the insertion of 1 (or 2) galactosyl β 1, 4 glucosaminyl disaccharides into the interior of the chain. The amounts of A^c and A^d are relatively small in the erythrocytes of the new born and in A₂ cells.

The glycolipids from human pancreas and small intestine contain chains where the galactose is 1 \rightarrow 3 linked to the N-acetylglucosamine rather than 1 \rightarrow 4 linked i.e.:

Hog gastric mucosa contains blood group glycolipid with a polygalactosyl core.

Proteins with blood group activity

Glycoproteins containing blood group activity are found in red cells and in a variety of other proteins. The glycopeptides or oligosaccharides obtained from digestion of these proteins react rather weakly with antibodies to blood group substances. Bands 3 and 4.5 carry the major activity. Some of the carbohydrate is connected to the protein through N asparaginyl links the rest by way of threonine or serine. Proteins and glycoproteins from other sources than red cells also contain blood group reactive carbohydrates. Komoda et. al., (Clin. Chim. Acta 117, 167-187 (1981)) found for example that one form of alkaline phosphatase from the intestinal mucosa of persons of type A blood group contained A antigen. The inner core of the antigen differs substantively from that of the glycolipid antigen. The relative contribution of the glycolipids and glycoproteins to antigenicity is unclear.

B. ENZYMES CONVERTING TYPE A BLOOD GROUP SUBSTANCE TO TYPE O (AZYMES)

Schiff (J. Inf. Dis. 65, 127-133 (1939)) was the first to report that culture filtrates of Clostridium welchii were capable of destroying the specific serological properties of blood group A-active substances. McGuire et al. (Methods in Enzymology 28, 755 (1972)) purified Azyme from the filtrate of Clostridium perfringens by ammonium sulfate precipitation, gel filtration, and DEAE-cellulose chromatography. The enzyme was stable for at least one year at -10°C. The optimal pH for enzyme activity was pH 5.8. Bell et al. (Carbohydrate Res. 61, 447-455 (1978) and Levy and Aminoff (J. Biol. Chem. 255, 11737-11742 (1980)) have reported further purification of this enzyme.

Enzymes of similar specificity have been partially purified from a variety of sources including the snail Helix pomatia (Tuppy and Staudebauer, Biochemistry 5, 1742 (1966)), the gastropod Turbo cornutus (Murematsu, ., J. Biol. Chem. (Tokyo) 64, 521 (1968)) and the limpet patella variegata (Uda et. al., J. Biol. Chem. 252, 5194 (1977)). The latter enzyme was purified 860 fold, had a pH optimum of 3.8 and an isoelectric point of 5.5. The enzyme was capable of liberating the N-acetylgalactosamine unit from Forssman hapten glycolipid, blood group A-active glycolipid, asialo bovine submaxillary glycoprotein, and blood group A-active glycoproteins.

Mammalian α -N-acetylgalactosaminidase was purified from pig and beef liver extracts by ammonium sulfate precipitation, DEAE-cellulose column chromatography, and gel filtration (Weissman and Hinrichsen, *Biochemistry* **8**, 2034-2043 (1969)). The enzyme was purified 300-fold from pig liver and 700-fold from beef liver. The pig liver Azyme exhibited maximum activity at pH 4.3 and was stable under acidic conditions. N-acetylgalactosamine was shown to be a competitive inhibitor with a K_i of 10.1 mM. The K_M for p-NP- α -GALNAc was 6.6 mM. In addition, isoelectric focusing revealed eight isozymes in the pig liver extract.

The beef liver Azyme exhibited optimal activity at pH 4.7 and a K_M for P nitrophenyl- α -N-acetylgalactosamine of 2.7 mM. The partially purified enzyme preparations from both beef and pig liver catalyzed the release of N-acetylgalactosamine from desialized sheep and beef submaxillary mucins. These residues are bound to the peptide core by α -glycosidic linkages. The enzyme also acted on blood group A substance isolated from pig stomach.

Porcine liver α -N-acetylgalactosaminidase was also isolated by Sung and Sweeley (*J. Biol. Chem.* **255**, 6589-6594 (1980)). The purification scheme included ammonium sulfate precipitation followed by successive chromatography on DEAE-cellulose, concanavaline A-Sepharose, DEAE-cellulose with ampholyte, elution, Sephadex G-150, and hydroxylapatite. Azyme activity for p-NP- α -GALNAc was improved 3,300-fold and the Forssman hapten hydrolyzing activity 19,600-fold by these procedures. Gel filtration on Sephadex G-150 indicated that the isolated enzyme was a dimer with a molecular weight of 102,000. The observed K_M for P nitrophenyl- α -N-acetylgalactosamine and the V_{max} 14.8 μ moles $\text{min}^{-1} \text{mg}^{-1}$. The enzyme was also tested with a variety of other substrates, including Forssman hapten ($K_M = 2.6$ mM, $V_{max} = 4.2$ μ moles $\text{min}^{-1} \text{mg}^{-1}$) and 4-methylumbelliferyl- α -galactopyranoside ($K_M = 0.01$ M, $V_{max} = 3.0$ μ moles $\text{min}^{-1} \text{mg}^{-1}$). The enzyme also hydrolyzed human erythrocyte A-active glycosphingolipids and a dog intestinal A-active glycosphingolipid.

Interest in Fabry's disease, a deficiency of α galactosidase A, sparked research in human Azyme which for many years was called galactosidase B until Dean et al. (*Biochem. Biophys. Res. Comm.* **77**, 1411-1417 (1977)) demonstrated its α -N-acetylgalactosaminidase activity and showed that it hydrolyzed Forssman hapten and globotriglycoylceramide. Its K_m for the N acetyl sugar substrate was 1/5 that for the galactosyl derivative.

Azymes have been prepared from human liver, spleen, placenta and plasma. The activity has also been found in brain and fibroblasts (Callahan et al., *Biochemical Med.* **7**, 424-431 (1973)). The liver enzyme has been purified by Ho (*Biochem. J.* **133**, 1 (1973)), Romeo et al. (*Biochem. Biophys. Acta* **391**, 349 (1975)), and Dean et al. (*J. Biol. Chem.* **254**, 10001-10005 (1979)). Ammonium sulfate fractionation, usually 30% to 60%, was used as a first step by all as a first step. DEAE cellulose chromatography was also used by all three groups. Their sequence of steps are indicated in the following chart.

Ho	Romeo et al.	Dean et al.
30-60% AMS	30-60% AMS	30-60% AMS
Sephadex g 150	Ethanol precipitation	DEAE cellulose
DEAE cellulose	ZaSO ₄ precipitation	Sephadex g 150
	DEAE cellulose	Hydroxylapatite
	Sephadex g 150	Ampholyte displacement
	Hydroxylapatite	

The purified Azyme extracted from liver was two to five-fold more active as an α -N-acetylgalactosaminidase than as an α -galactosidase. The hydrolysis of synthetic substrate was optimal at pH 4.3 and the K_m was 1.3 ± 0.1 mM. The enzyme was shown to catalyze the hydrolysis of several water-soluble oligosaccharides derived from globotri- β -glucosylceramide and globopentaglycosylceramide (Forssman antigen). This led to the suggestion that the role of Azyme *in vivo* was the hydrolysis of glycoconjugates containing terminal α -N-acetylgalactosamine residues such as Forssman antigen and group A-active glycolipids and glycoproteins. A spectacular purification of human liver α -galactosidase was obtained by Bishop et al., who purified the liver enzyme 220,000 fold. They used α -D-galactopyranosylamine coupled through a long spacer to Sepharose. This affinity procedure was also used for purification of the enzyme from placenta and spleen. Azyme also adsorbs to the affinity column and must be separated from α -galactosidase A by prior DEAE chromatography. This affinity procedure has not been applied to Azyme but should be very useful.

Placenta has served as the other major source of human Azyme. Beutler and Kuhl (J. Biol. Chem. 247, 7195-7200 (1972)), Johnson and Brady (Methods in Enzymology 28, 849 (1972)), Geiger et al. (Eur. J. Biochem. 56, 311-318 (1975)), Geiger et al. (Biochemistry 15, 3484-3493 (1976)), Kusiak et al. (J. Biol. Chem. 253, 184-190 (1978)) and Mahuran and Lowden (Canad. J. Biochem. 58, 287-294 (1980)) are among the groups who have developed purification procedures.

Beutler et al.	Kusiak et al.	Geiger et al.	Mahuran et al.
DEAE cellulose	Con A Sepharose	Con A sepharose	Con A sepharose
35-50% AMS	DEAE cellulose	DEAE cellulose	Affinity column
g 200 Sephadex	Sephadex g 200	Sephadex g 150	DEAE sepharose
Electrofocusing	SP sephadex		
Ecteola cellulose	Butyl agarose		
3990	39000	5100	11000

The method of Kusiak et al. superseded that of Johnson and Brady. The affinity column used by Mahuran et al. was also used by Geiger et al. and was 2 acetimido-N-Eaminoproyl-2-deoxy glucopyranosyl amine coupled to Sepharose 4B (Geiger et al.) or Sephacryl S-200 (Mahuran et al.).

Azyme appears to be a heat stable homotetramer which has a molecular weight near 100,000. Beutler et al. found an isoelectric point 4.42 and a pH optimum for 4 methylumbelliferyl- α -D-galactopyranoside of 4.5. Kusiak et al. suggested that blood group B or A substances might be the natural substrate for the enzyme.

Mahuran et al. and Geiger et al. (Biochemistry 17, 1713-1717 (1978)) have studied an additional form of Azyme. The former obtained it from placenta by treating galactosidase A with merthiolate. Geiger et al. obtained it from pregnancy serum. The form prepared by Mahuran et al. is believed to have the structure (α_1) where α_1 is one of the chains found in galactosidase. The material of Geiger et al. possessed α -N-acetylgalactosaminidase (Azyme) activity. Mahuran and Lowden used 4-methylumbelliferyl- β -D-N acetyl glucosamine as substrate. The relationship between the two "isozymes" is unclear. Schram et al. (Biochem. Biophys. Acta 525, 410-416 (1978)) have shown that human liver Azyme undergoes a conversion to a second form with lower molecular weight and slightly changed kinetic properties upon aging.

II. Purification of Bacterial Azyme

Isoelectric focusing was studied as a method for purifying bacterial Azyme. In this procedure a pH gradient is created by applying an electric field to a complex mixture of amphoteric substances (ampholytes) which line up according to their isoelectric points. Proteins are usually introduced at the alkaline end of this gradient and migrate until they reach their isoelectric point.

The bacterial Azyme used was a crude preparation from *Clostridium perfringens* culture broth. It was supplied by Dr. David Aminoff of the University of Michigan. In addition to Azyme (α -N-galactosaminidase) it contained considerable β galactosidase and α -N-acetylglucosaminidase activity. These three enzymatic activities were monitored to determine optimal separation.

A. METHODS

Preliminary studies were done in polyacrylamide gel for economy of enzyme and reagents and since large amounts of enzyme were not available initially.

Polyacrylamide Gel Isoelectric Focusing

Acrylamide of electrophoresis purity from Eastman, N,N' - methylene - bisacrylamide (BIS) of electrophoresis purity from Bio-Rad Laboratories and N,N' - diallyltartardiamide (DATD) from Eastman, were used without further purification. For some experiments acrylamide (Baker) twice recrystallized from CHCl_3 was used. Wide range (pH 3.5 - 10) ampholyte was prepared by the copolymerization of acrylic acid with pentaethylene hexamine (PEHA) as described by Vinogradov et al. (Biochem. Biophys. Res. Comm. 54:501-506 (1973)), with the exception that initially PEHA was not vacuum distilled but rather was passed through a charcoal column. The PEHA ampholyte was passed through a millipore filter, made up to 40% (w/v) with distilled water and stored at -20°C prior to use.

Narrow range, neutral (pH 6 - 8) Ampholine, Lot 3, as well as broad range (pH 3.5 - 10) Ampholine, Lots 16, 17 and 29 were purchased from LKB.

Acid washed glass tubes were used. Separators were added prior to polymerization with ammonium persulfate. Water was layered carefully on top of the gel to prevent oxygen inhibition and insure a level gel surface. Polymerization was performed at room temperature and took approximately 5 - 10 minutes. Unless otherwise indicated the cylindrical gels containing BIS as crosslinker were 6% T, 3% C; those containing DATD were 5% T, 15% C, where:

$$\% T = \frac{\text{gms acrylamide} + \text{gms crosslinker}}{100 \text{ ml gel solution}}$$

$$\% C = \frac{\text{gms crosslinker} \times 100}{\text{gms acrylamide} + \text{gms crosslinker}}$$

Gels were cooled at 4°C for at least 30 minutes prior to use.

Gels were removed from the cold room and Parafilm covering the bottoms of the gels was also removed. The bottom of the tubes was then covered with a paper cloth and secured with a rubber band. Cheese cloth or dialysis tubing could also be used. The purpose of this was to support the gels to prevent slippage during focusing. Water was added to the tops of the gels, up to the top of the glass tubes.

The anolyte was 0.01 M phosphoric acid and the catholyte was 0.02 M sodium hydroxide unless otherwise noted. For experiments with amino acids as ampholyte the anolyte was 0.01 M aspartic acid and the catholyte was 0.01 M lysine.

The gels were run at 0 - 4°C at 1 ma/tube until a voltage of 150 volts was obtained and this voltage was maintained for approximately 18 hours, unless otherwise noted. The gels were prefocused at least one hour prior to sample application. Protein solutions containing sucrose were added onto the top of the gels with a Hamilton syringe.

For most experiments 10 cm gels were cast in 6 mm diameter tubes. At the termination of the experiment the gel was removed from the tube and cut into 2 mm thick slices. The slices were each placed in 0.5 ml of 25 mM KCl (boiled for five minutes). The tubes were allowed to incubate for one hour at room temperature. The pH gradient was then determined by pH measurements on the diffusate of each consecutive gel slice and 0.3 ml of 0.5 M phosphate buffer, pH 6.0, was added to each tube. The slices soaked overnight at 0°C and aliquots of the diffusate were assayed for enzyme activity.

Enzyme Assays

Azyme activity was measured in a 250 μ l assay system containing 0.1 M buffer, pH 6.0, 0.36 mM p-nitrophenyl-2-acetamido-2-deoxy- α -D-galacto-pyranoside (Koch-Light Laboratories), distilled water, and appropriately diluted enzyme. The assay was incubated for one hour at 37°C (unless otherwise stated) and the reaction was terminated by the addition of 200 μ l of 15% Na₂CO₃. The absorbance at 400 nm was determined in a Zeiss PMQ III spectrophotometer. Assays for β -N-acetylglucosaminidase (GNase) and β -galactosidase (GALase) activity were performed in a 250 μ l assay system containing 0.5 M sodium phosphate buffer pH 6.0 (when diluted 1:5), distilled water, and the appropriate substrate. The substrates employed were 0.36 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (Calbiochem) for GNase assays and 0.41 mM p-nitrophenyl- β -D-galactopyranoside (Calbiochem) for GALase assays. The assays were incubated for 15 minutes at 37°C and the reaction terminated by the addition of 200 μ l of 15% Na₂CO₃. The absorbance at 400 nm was recorded.

B. ISOELECTRIC FOCUSING UNDER STANDARD CONDITIONS

In the presence of a wide range ampholyte synthesized in our laboratory, the Azyme focused near pH 6.7. The enzyme focused at pH 6.74 in commercial ampholyte (LKB pH 3.5 - 10 Ampholine Lot 16). The pH of focusing was unaffected by running for 48 instead of 24 hr, suggesting that the focusing was complete. The focusing was reproducible. The mean pI from eight runs was 6.70. All of the pIs were within ± 0.13 of this value. In general, the contaminating enzymes, galactosidase and N-acetylglucosaminidase, focused very near the Azyme (Figure 1).

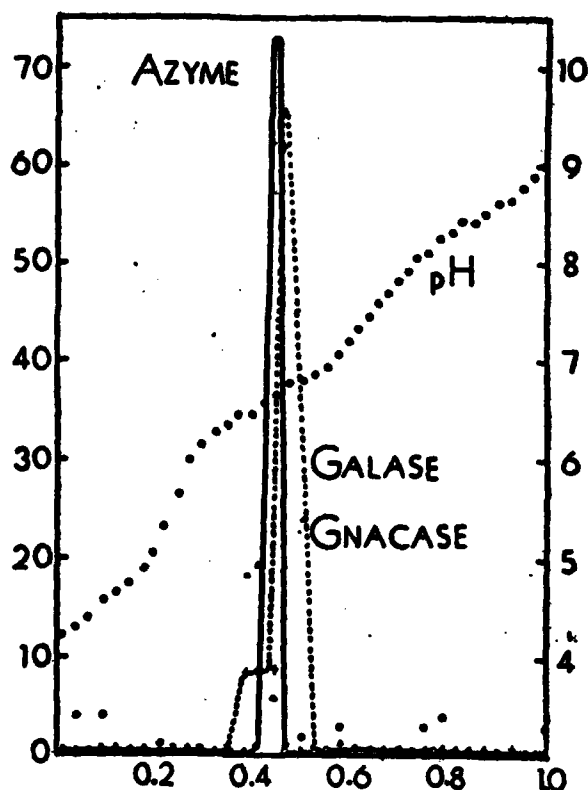


Figure 1. Isoelectric focusing of crude Azyme (o—o) in 6% acrylamide gel 15% crosslinked with diallyl tartardiamide and containing 2% ampholyte. The contaminating enzymes, β -galactosidase, GALase, (Δ — Δ) and α -N-acetyl glucosaminidase, GNACase, (+ — +) are poorly separated.

C. SEPARATOR ISOELECTRIC FOCUSING

In the first series of experiments we tried to increase the resolution by flattening the pH gradient. We had previously used this procedure quite successfully for the separation of the minor sub-types of human hemoglobin, goat and human antibodies, and barley β -amylase (Caspers, Posey and Brown, Anal. Biochem. 79, 166-180, 1977). The flattening is accomplished by adding separators, small amphoteric molecules such as amino acids with appropriate isoelectric points. In the case of Azyme, histidyl glycine (Vega-Fox) 5-aminovaleric acid, (Aldrich) 6-aminocaproic acid (Mann), histidyl phenylalanine (Cyclol), histidyl histidine (Vega-Fox), histidyl proline (Chemalod) and mixtures of histidyl phenylalanine and histidyl glycine and of 6-aminocaproic acid and histidyl glycine were tried. Focusing in histidyl glycine separated the galactosidase activity into 2 peaks but did not separate the Azyme from the contaminants (Figure 2). The apparent isoelectric point of the Azyme was altered from 6.70 to 5.77.

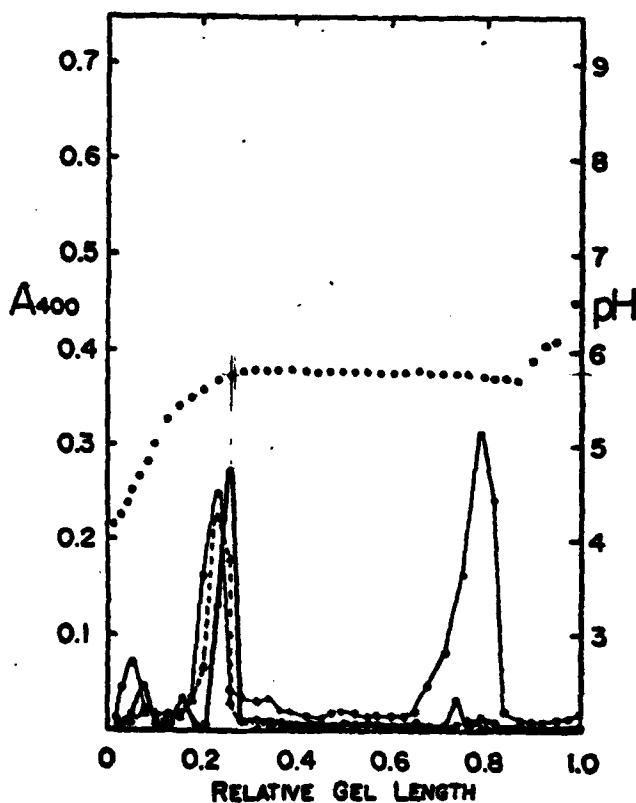


Figure 2. Isoelectric focusing of crude Azyme in an acrylamide gel containing histidyl glycine (10 mg/ml) as separator. Note flatness of the pH gradient in comparison with Fig. 1. Although there was separation of the galactosidase (o—o) activity into 2 peaks, the Azyme (●—●) was contaminated with both galactosidase and glucosaminidase (Δ — Δ).

D. FOCUSING WITH NARROW RANGE AMPHOLYTES

In the next group of experiments we attempted to alter the pH gradient by using narrow range ampholytes. Commercial products fractionated from long range ampholytes were used in the experiments. Mixtures of LKB pH 5-8 ampholyte (Batch 46) with histidyl phenylalanine with or without (Figure 3) added LKB pH 4-6 ampholyte (Lot 7) were used. In these systems the Azyme focused at pH 4.97 \pm .05. The shift in apparent isoelectric point was quite unexpected and a number of experiments were done to determine its basis since utilization of this phenomenon might form an excellent basis of purification.

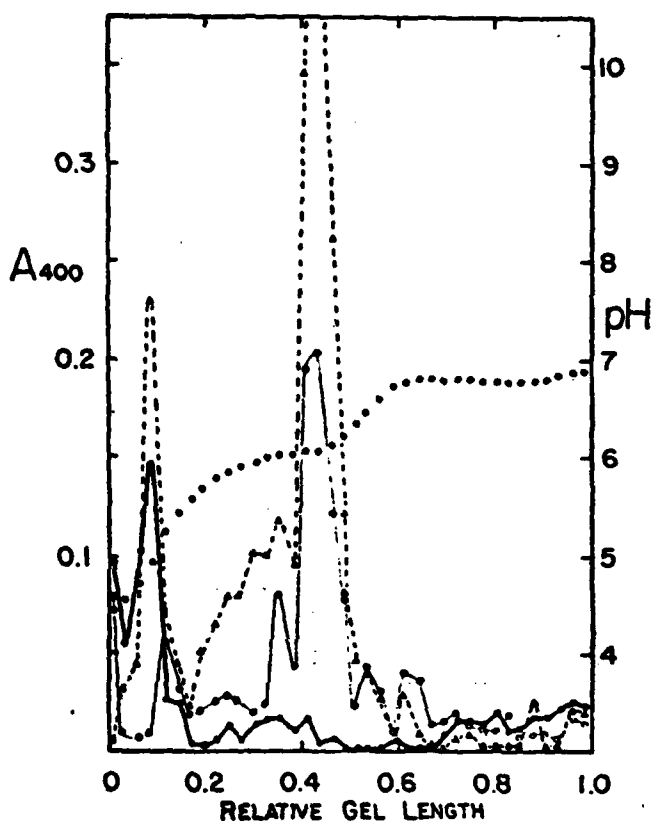


Figure 3. Isoelectric focusing of crude Azyme in an acrylamide gel containing LKB pH 5-8 Ampholine (Lot 43) and histidyl phenylalanine (6 mg/ml). The apparent pI of the Azyme was 4.92. The separation of Azyme (●—●) from glucosaminidase (Δ—Δ) and galactosidase (○—○) was improved.

E. INTERACTIONS OF AZYME

In one group we tried to determine if the unusual isoelectric focusing was due to interactions of the enzyme with other impurities in the system. Three different crude preparations were studied as well as a sample of Azyme which had been partially purified by gel filtration. All exhibited similar behavior. Other proteins were added to the system to see if they changed the behavior. Of these, only lysozyme, which is a small basic protein, interacted with Azyme. Treatment of crude enzyme with mild acid (10 days at 4°C in 0.1 M pH 5.03 acetate buffer) or with 3 volumes of acetone at room temperature (Schiff, F., J. Inf. Dis. 65, 127, 1939) might be expected to alter labile impurities and their interactions. Neither treatment altered the focusing pattern. The acid treated enzyme focused in a time independent manner between 17-1/2 and 34-1/2 hr. Enzyme which was focused, eluted, and refocused, refocused near the original pH (Figure 4).

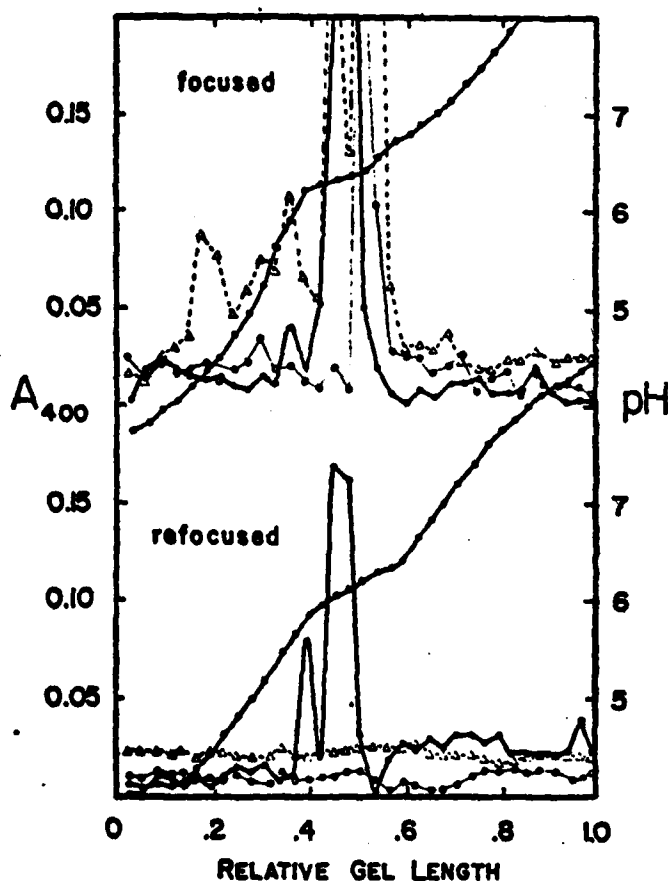


Figure 4. Refocusing of Azyme. Crude Azyme (.36 mg, 7 times the usual load) was focused in a 6% acrylamide 15% crosslinked gel in 2% PEHA I ampholyte. After 20 hr the gel was sliced and assayed for Azyme (o—o), galactosidase (Δ — Δ) and glucosaminidase (o—o) (top graph). One half of the slice containing the maximal Azyme activity was dispersed in 1/2% PEHA I, placed on top of a new gel and refocused (bottom graph). The Azyme refocused near its original position and was free of contaminating enzymes.

The refocused enzyme appeared free of impurities suggesting that this is one method of purification. These findings suggested that interaction with other protein components in the crude enzyme was not the cause of the unusual focusing.

A number of disc electrophoresis experiments were done to determine whether the Azyme was interacting with an ampholyte component or any of the substances used as separator. None indicated any interaction. If the Azyme were interacting with ampholyte components, the focusing pattern would be expected to differ depending on whether the components were prefocused or not. The focusing was the same in both experiments. These findings suggested that interaction with small molecules was not responsible for the anomalous focusing. On the other hand, focusing the Azyme in the long range ampholyte in the presence of glutamic acid gave acidic apparent isoelectric points which appear to depend partially on the amount of glutamic acid used. At 5 mg/ml the pIs were 6.05 and 5.60, at 8 mg/ml 4.9 and 4.3, and at 10 mg/ml 4.45. If the sample was focused in long range ampholyte and glutamic acid (25 mg, i.e., 8.3 mg/ml) was added after focusing the pI was 3.82.

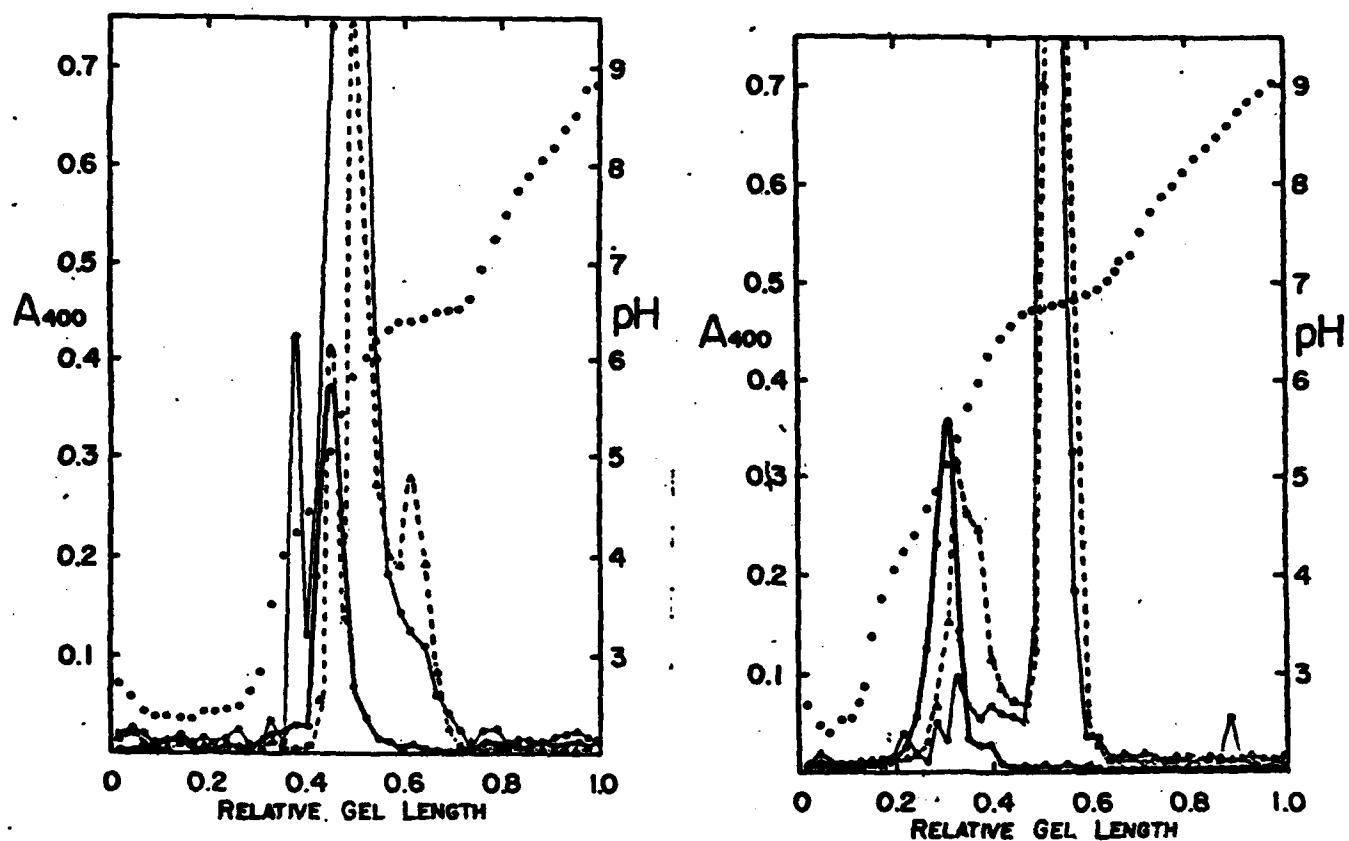
The stability of the enzyme was examined under the conditions of focusing to see if changes in its activity could explain some of the findings. The stability of crude Azyme was examined at pHs between 3.5 and 6.0 in 0.01 M acetate buffers. The enzyme was fairly stable, 88% of the activity remained after 24 hr at 4°C. An additional stability study at pH $3.69 \pm .03$ and 4° indicated that sodium chloride at concentrations of 0.01, 0.05 and 0.10 did not alter the stability of crude Azyme over a 50 hr period.

Since we obtained different isoelectric points in the isoelectric focusing experiments it seemed worthwhile to determine which was the true value. In disc gel electrophoresis at pH 8.5 and 7.0 the enzyme has an isoelectric point which is quite acid to pH 7. An electrophoresis at pH 3.62 suggested that the isoelectric point is alkaline to this. In this experiment the enzyme was layered in the middle of the gel. It moved slightly toward the negative pole but the movement was not great. Insolubility of the enzyme may have affected the results.

F. OPTIMIZATION OF SEPARATION

While we were attempting to find the basis for the unusual focusing behavior we proceeded simultaneously to empirically optimize separation conditions. Focusing for longer or shorter times (21, 27, 58, 56 hr) in short (10 cm) or long (60 cm) columns (Rapaport, R., Jackiw, B.A., and Brown, R.K. Electrophoresis 1:122-126 (1980)) at higher or lower voltage or with up to 7 times the usual load of enzyme, thawing, freezing or warming the enzyme, did not influence the separation. The 60 cm columns allow us to obtain a very shallow pH gradient (0.01 pH unit/cm).

The separation depended upon the concentration of carrier ampholyte. Using ampholyte made in our laboratory the separation steadily improved as the concentration of ampholyte was increased from 3/4% to 2%, to 8% (Figures 5, 6 and 7). Azyme was purified 538-fold in relation to galactosidase and 428-fold compared to glucosaminidase in the experiment shown. The character of the ampholyte was also critical. This is discussed in a subsequent paragraph.



Figures 5, 6. Effect of ampholyte concentration on the separation of Azyme from its impurities. Ampholyte concentration of 3/4% (Figure 5), or 2% (Figure 6). Total acrylamide concentration 5% with 15% diallyl tartar-diamide as crosslinker. Azyme (●—●), pH gradient (● ●), galactosidase (Δ—Δ), glucosaminidase (○—○).

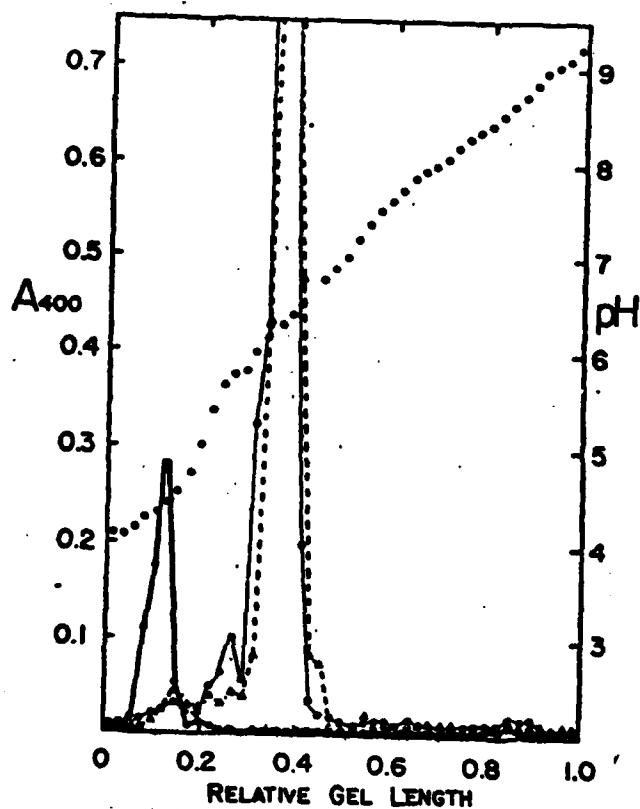


Figure 7. Effect of 8% ampholyte on Azyme purity. Total acrylamide concentrations 5% with 15% diallyl tartardiamide crosslinker. Azyme (●—●), pH (● ●), galactosidase (Δ—Δ), glucosaminidase (○—○).

Total acrylamide concentration also affected the separation. In these experiments total acrylamide concentration was varied from 5% to 4% with the % crosslinker being held constant at 15%. Better separation was obtained at lower acrylamide concentrations. Although the effect of the nature of the ampholyte was not studied at this time, later studies described in a following paragraph indicated that the separation depended strongly upon the ampholyte preparation.

G. ISOELECTRIC FOCUSING IN SEPHADEX

Following the demonstration that Azyme could be separated from its contaminating enzymes on an analytical scale, we turned to modifying the procedure so that larger amounts of material could be processed. For this it was necessary to first change the support medium from polyacrylamide gel to G75 superfine Sephadex. The latter is a bead formed crosslinked dextran gel which has been widely used for preparative isoelectric focusing. Although our intent was to use thin sheets of this medium in a flat bed focusing apparatus, preliminary experiments were done with the 12 x 0.6 cm tubes used for gel isoelectric focusing. This provided economy of enzyme and ampholytes. The fine granules of Sephadex are held in the tube by a 1 cm segment of polyacrylamide gel. The gel (16% acrylamide, 3% crosslinked with bisacrylamide) has a small pore size which should prevent the enzyme from leaving the tube.

Initial experiments were under the conditions which had given good separations in gel tubes except that it was necessary to change ampholyte lots since PEHA I was in short supply. The separations were much poorer than in the gel system and several factors were examined to see their influence on the separation process. In experiments in polyacrylamide gel it was found that neither PEHA III (Figure 8) nor LKB 3.5-10 ampholyte gave good separation of Azyme from impurities although PEHA I did. A time study using LKB 3.5-10 ampholyte in gel showed a slight improvement in focusing with time in a set of samples examined at intervals of 3 hr over a period of 18 hr. But the resolution was still inferior.

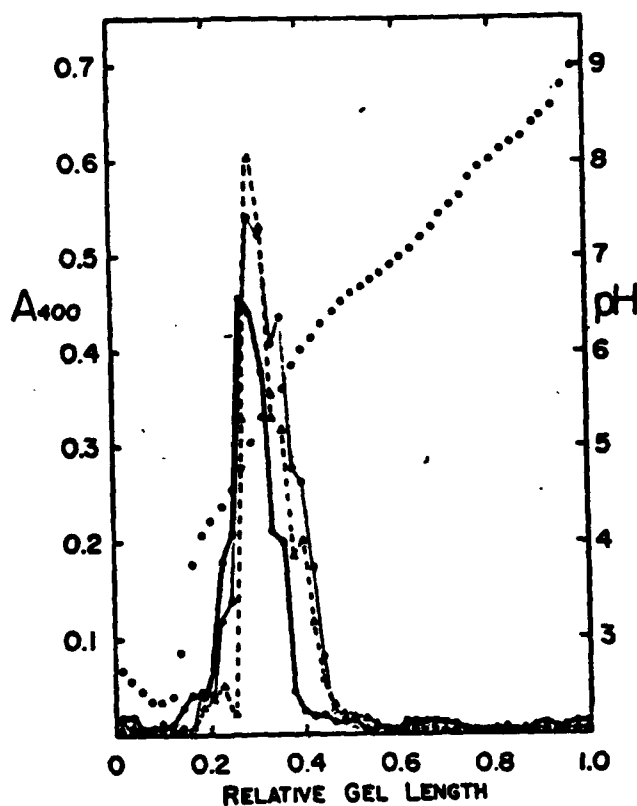


Figure 8. Effect of ampholyte preparation on Azyme purification. The conditions are the same as those in Figure 6 except for the ampholyte used. PEHA III was used here while PEHA I was used in the previous experiment.

H. INSOLUBILITY OF AZYME

These facts were in large part resolved by the observation that Azyme is insoluble in focused ampholyte, water, or 0.05 M KCl over a considerable pH range near its isoelectric point. It is soluble in 0.25 M KCl. These observations could not have been made with the gel system where entrapment in the gel matrix is not easily differentiated from insolubility. The amount of material is so small that there is no visual indication of precipitation. In the Sephadex the processes are readily delineated. These findings suggest that the separation observed in analytical gels depends on both isoelectric focusing and insolubility. The Azyme and impurities separate partially or completely during focusing and then become insoluble. This insolubility extends over an appreciable pH range. The time the enzyme is exposed to a given pH, the porosity of the gel, the amounts of separators and type ampholyte components would all be expected to influence precipitation as is inferred from the data. If a shift in the pH gradient occurs, the insoluble Azyme remains at the location of its original precipitation although the pH of that region changes. This probably accounts for the various isoelectric points obtained under different experimental circumstances.

I. EFFECT OF SOLUBILIZING AGENTS

Isoelectric focusing was done under conditions where the Azyme would remain soluble. In addition to solubilizing the enzyme, salt also has salutary effects on the potential gradient of the system. Jackiw and I (Electrophoresis 1:107-113 (1980)) have measured the changes in the potential gradient along the tube during focusing. As focusing continues much of the potential drop occurs in one small region of the tube causing local heating and leaving such a small potential drop across other regions of the tube that the focusing is poor. Salt corrects both the solubility and potential gradient problems.

Isoelectric focusing in salt solutions was explored in several ways. In one, a sample was focused in the normal manner. Then the electrolyte solutions were replaced by those containing salt. After the solubilization was complete as judged by appearance (in heavily loaded system) and current measurements, electrolytes containing salt were replaced by electrolytes of the original sort (.02 M NaOH and 0.01 M H_3PO_4) and focusing was continued. In the second type of experiment the entire system contained the salt and in the third sort of experiment the salt was in the tube containing the sample but not in the electrolytes. In many cases samples were applied to both the top and the bottom of separate gels. If focusing occurs the outcome should be independent of the site of sample application. The best Sephadex separation to date in a system involving salt was under the following conditions: G75 superfine Sephadex gel loaded from bottom Ampholyte, Pharmalyte pH 2-5, 1.33% LKB 4-6 ampholyte 0.6%, running voltage 250 min at 25 volts, 808 min at 50 volts, 113 min at 75 volts, 109 min at 100 volts. Both Sephadex and electrolytes contained 0.15% KCl. The data (Figure 9) plotted is a two-point running average of the observations.

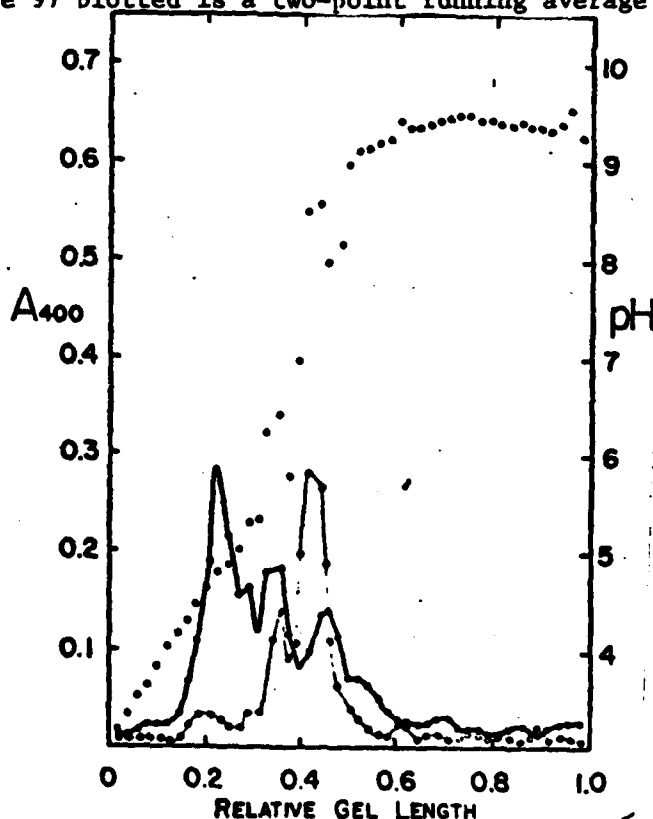


Figure 9. Isoelectric focusing of Azyme on Sephadex G75. Ampholyte pharmalyte pH 2.5-5; 1.33%; LKB Ampholine 0.67%. Both column and electrolytes contained 0.15 M KCl. Azyme (●—●), pH (○...○), galactosidase (○- -○). Considerable separation was achieved although the purity was less than obtained in analytical isoelectric focusing.

A number of solubilizing agents were examined to see if they inhibit Azyme activity. Urea, at a concentration of 1 M gave 27% inhibition and at 2 M the inhibition was 55%. Triton, a non-ionic detergent, had little effect on the activity at concentrations of 0.1 and 0.5%. Sulfobetaine, at 0.1, 0.2, 0.5 and 1.0 did not change the activity. Sodium dodecyl sulfate inactivated the enzyme even at 0.05%.

III. Preparation and Properties of Placental Azyme

The major thrust of the second phase of the project was to develop a human source of Azyme. Previous work employed enzymes from bacteria. Even though the erythrocytes are treated in vitro and the enzyme is removed, it is difficult if not impossible to rule out the presence of small amounts of bacterial protein contaminating the erythrocytes. These might give rise to serious immunologic reactions such as anaphalaxis. Using enzymes from humans avoids this problem. Placentas were chosen as a source of this enzyme because of their availability and because they have long been used as a source of plasma for fractionation for human use.

A. ENZYME ASSAY

Placental azyme activity was measured in a 250 μ l assay system containing 1 M sodium acetate buffer, pH 4.0, 0.36 mM p-nitrophenyl-2-acetamido-2 deoxy- α -D-galactopyranoside (Koch-Light Laboratories) for 1 hr at 30°. The reaction was terminated with 200 μ l of 15% Na_2CO_3 and the absorbance at 400 nm read in a Zeiss PMQ III spectrophotometer. For crude fractions it was modified to remove interfering color from hemoglobin by precipitating protein after incubation. For this the procedure was modified by termination of the assay reaction with the addition of 100 μ l of 50% trichloroacetic acid in 1 M NaCl. The precipitate was collected by centrifugation for three minutes in an Eppendorf microcentrifuge. A portion of the supernatant (175 μ l) was removed, neutralized with 300 μ l 2 N NaOH and mixed with 140 μ l 15% Na_2CO_3 . The absorbance was determined at 400 nm as previously. The assay is reproducible and linear.

B. COLLECTION AND STORAGE OF PLACENTAS

Placenta contains a large amount of blood. Accordingly cord blood was examined to see if it contained Azyme. Bloch, Betschart and Burger (Exp. Cell Res. 104, 143-152 (1977)) reported finding α -N-acetylgalactosaminidase activity in fetal calf serum although adults possessed only trace amounts. On the possibility that newborn humans would possess such activity we examined samples of fresh cord blood for Azyme activity. No activity was found in assay of eight cord blood samples from humans. Under the assay conditions the sera gave a 50% inhibition of Cl perfringens Azyme activity. To help rule out the possibility that Azyme activity was present but inhibited, one sample was separated by isoelectric focusing which should separate putative inhibitor and Azyme. No Azyme activity was found in the focused sample. Since the Azyme is in the placental tissue itself, attempts were made to perfuse the placentas to remove the blood. These were unsuccessful. An expert in placental physiology was consulted. He felt that perfusion would be successful only if conducted within 15 minutes of delivery and with warm saline. This appears unfeasible and all work was done with non-perfused placentas.

A placenta obtained at the time of delivery was divided into 2 portions. One portion was homogenized and assayed within 1 hr. The other part was

frozen at -18° overnight and then assayed. The frozen placenta had a slightly greater activity indicating that placental Azyme survives freezing and thawing. Long term storage of frozen placentas did result in loss of activity. A placenta stored for 5 months at -18°C lost about 50% of its activity. The protein was also somewhat less extractable (15%) so that the homogenate made after 5 months of storage had about 60% of its original specific activity. Long term storage of placentas was avoided.

Purification of Placental Azyme

Placental Azyme was purified by ammonium sulfate fraction, isoelectric precipitation of impurities, and chromatography on DEAE cellulose and hydroxal-apatite. In all, over 100 placentas were fractionated although some of the material awaits chromatography. The studies listed delineate some of the properties of the enzyme which form the basis for the purification.

Use of Proteinase Inhibitors

Several investigators have used inhibitors of proteolytic enzymes to help stabilize the enzyme. The effect of phenylmethyl-sulfonyl fluoride was examined. It is a general inhibitor of the group of proteolytic enzymes which use serine as part of their catalytic site. Homogenates prepared in the absence of inhibitor have 82% as much activity as controls. Phenylmethylsulfonyl fluoride (0.5 mM) was used in all subsequent homogenizations.

Ammonium Sulfate Fractionation

A large number of concentrations of ammonium sulfate, both neutral and acid, at room temperature and at $+4^{\circ}$ were examined to determine the optimal conditions for fractionation. The pH and temperature were not critical variables. The fraction which precipitated between 36% and 62% ammonium sulfate saturation at $+4^{\circ}$ was purified 4.1 fold in a yield of 94% and was used for further studies.

Precipitation of Impurities

When the ammonium sulfate was removed by dialysis a copious precipitate appeared. It was essentially devoid of activity. Precipitation conditions were optimized by examining the influence of pH on the precipitability of the impurities. A series of 8 pH's between 4.91 and 6.80 were examined. A pH of 5.4 was selected. Under large scale conditions it gave a yield of 76% for the step and a purification of 2.5 fold.

Ethanol Fractionation

A wide variety of conditions were explored for ethanol fractionation. These included ethanol concentration (5% increments from 0 to 50%), pH (4.4 and 5.4), temperature (-20° , 0° and room temperature), and ionic strength (0 and 0.02 M acetate buffer). The activity remained in the supernatant. The purity was 5 fold and the yield 111%. These appear to be the optimal conditions for further work. While these studies were ongoing we wished simultaneously to undertake the purification by DEAE chromatography. For this purpose we fractionated at pH 5.4 in 50% ethanol at room temperature. After removal of the precipitate, the supernatant was placed at -18° for 2 hr after which it was recentrifuged. This precipitate was 5.7 fold purified but the

but the yield was only 31%. It was devoid of β galactosidase or β -N-acetylglucosamidase activity under the conditions of assay. The yield could not be improved and it was felt that it was too poor to include ethanol fractionation as a fractionation step.

Chromatography on DEAE

The placental Azyme was further purified by chromatography on DEAE cellulose on a 1.5 x 30 cm column of DEAE cellulose which had been equilibrated with 10 mM phosphate buffer at pH 6.5 at 4°. The enzyme was eluted by a 200 ml linear gradient which went from 0.0 to 0.5 M in NaCl. The gradient was started after 78 ml of buffer had passed through the tube. The Azyme appeared as a sharp peak at 178 ml, i.e., 0.25 M NaCl (Figure 10).

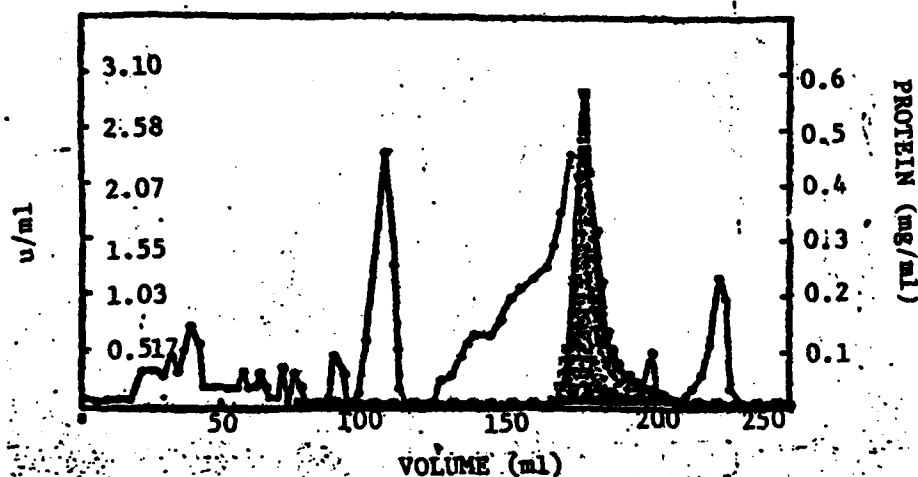
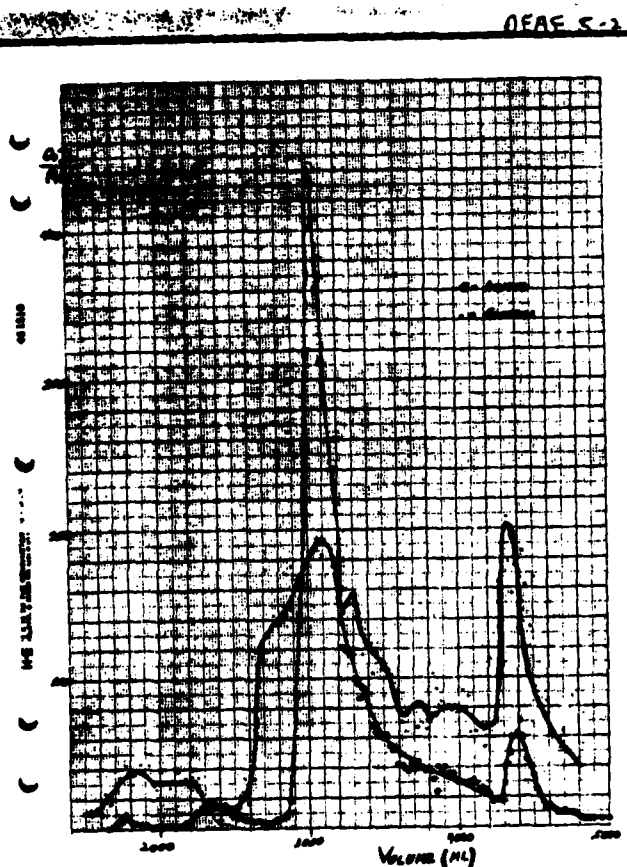


Figure 10. Small scale chromatography of placental Azyme (shaded area) on DEAE cellulose.

After a number of small scale runs, a large scale purification of Azyme on DEAE cellulose was developed using a 5 x 90 cm column loaded with 20 to 25 g of partially purified enzyme which had been prepared by ammonium sulfate fractionation followed by precipitation of impurities at low ionic strength. The Azyme was eluted with a linear gradient of sodium chloride going from 0 to 0.5 M at pH 6.5 in 0.01 M phosphate buffer. A total of 6.6 liters was used for elution. In addition to the major peak, 3 minor peaks were found (Figure 11). Further studies on the minor fractions are described later in the report.



Chromatography on Hydroxal Apatite

Due to limited funds available we attempted to prepare hydroxylapatite. Two methods of preparing hydroxylapatite were used, the Bernardi (Methods in Enzymology, 21, 96-147 (1971)) modification of the Tiselius procedure and the Spencer (J. Chromotogr. 166, 435-446 (1978)) method. The resolution and flow rate differed for the various hydroxylapatite preparations. Although adequate separation was secured the flow rate was extremely slow and commercially prepared hydroxylapatite (Calbiochem Fast Flow Hydroxylapatite 391947) was used in the preparative experiments. Several small scale runs were done with hydroxylapatite. In a typical run, a 1.0 x 14 cm column was loaded with 5.6 mg of protein and eluted with a 40 ml gradient of sodium phosphate buffer going linearly from 1 mM to 50 mM (Figure 12).

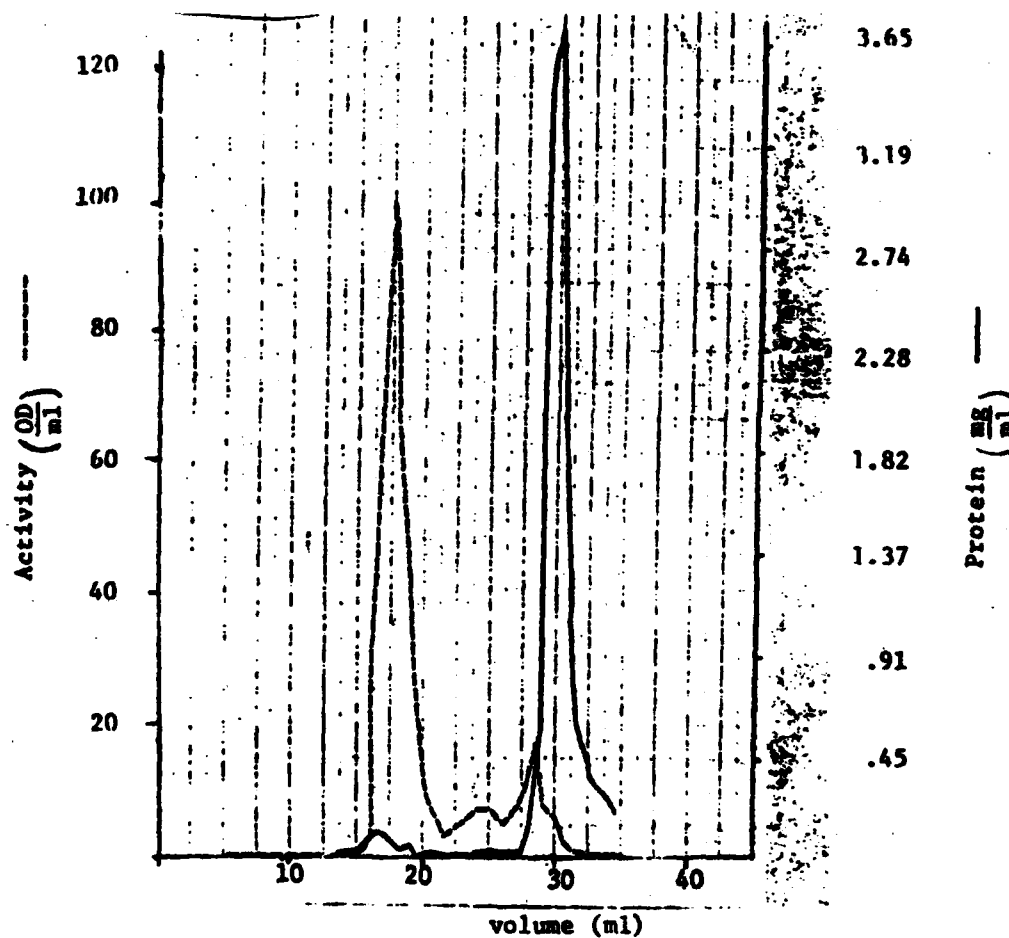


Figure 12. Purification of Azyme on hydroxylapatite enzyme activity (-----) and protein (——) are plotted against elution volume. Enzyme is purified 4600 fold over starting placental homogenate.

In a typical preparative run 582 mg of Azyme from DEAE chromatography was placed on a 2.5 x 28 cm column of regenerated hydroxy apatite. After 300 ml of 1 mM sodium phosphate pH 6.5 buffer passed through the column, a linear gradient going from 1 mM to 50 mM buffer was used. The flow rate was 20 ml/hr and 2 ml fractions were collected. The separation was in good agreement with the small scale runs although there is either skewing (perhaps from overload) or an additional component in the main peak. The minor components are more clearly discernable than in the small scale run (Figure 13).

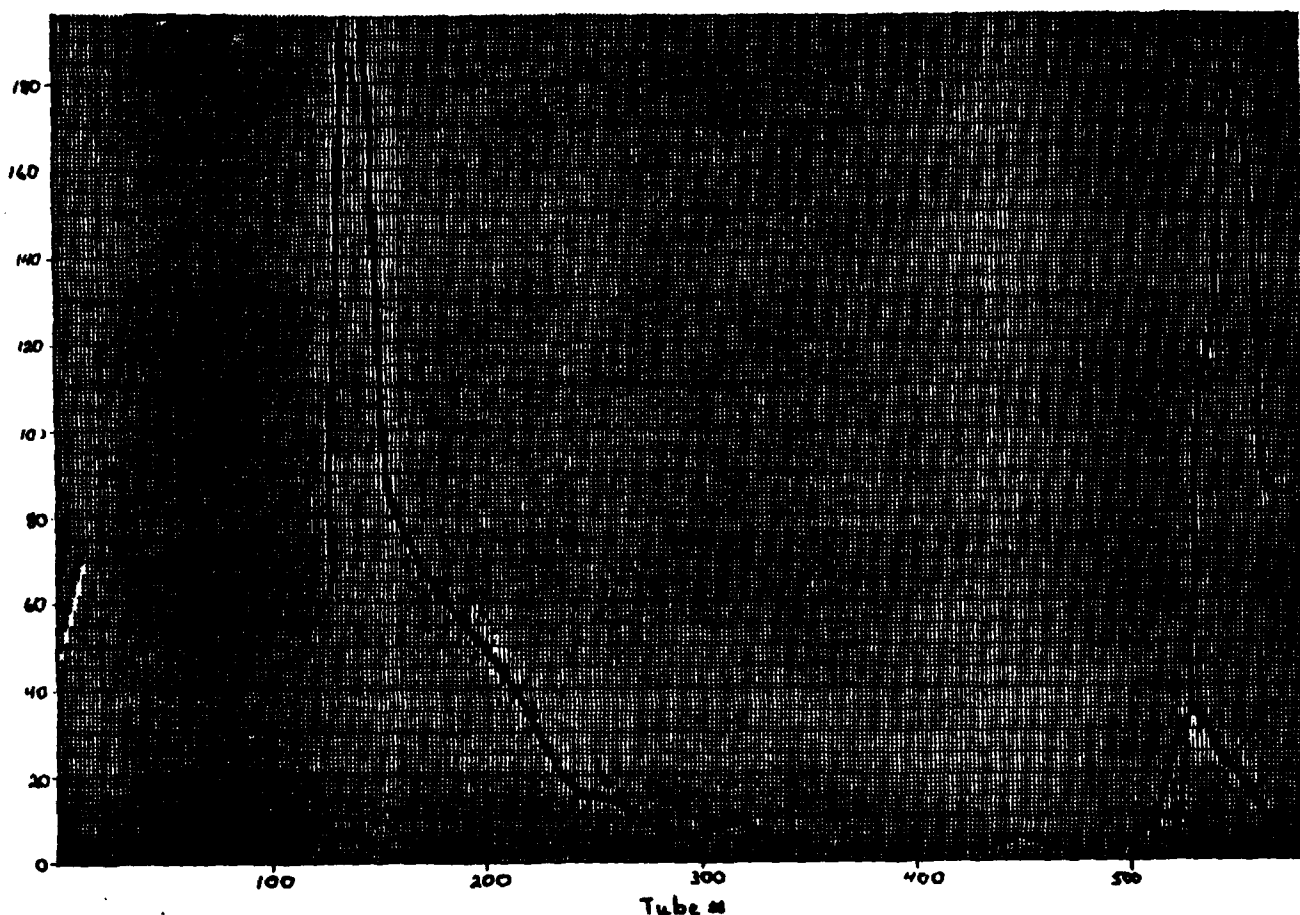


Figure 13. Preparative chromatography of Placental Azyme on a 2.5 x 28 cm column of hydroxylapatite. Conditions are described in the text. Protein (— — —), Azyme (— — —).

Experiments were also done to determine if batchwise elution would be successful. One ml of hydroxyl apatite was mixed with 0.5 ml of euglobulin supernatant (16.4 mg) and eluted successively with 2 washes of 2 ml each of the following pH 6.5 phosphate buffers. 1 mM, 5.5 mM, 10 mM, 25.5 mM, 50 mM, 100.5 mM and 200 mM. Thirty-five percent of the Azyme eluted at 25.5 mM with a specific activity of 291 u/mg and another 22% was in the second wash (specific activity 181 u/mg). The 2 fractions eluted at 10 mM each contained 9.5% of the material. The first elution at 50 mM contained 16.5% and the second 8% of the activity. The batchwise procedure is more convenient than the chromatography but the purification is much lower.

Concentration of Placental Azyme

In their description of the conversion of B erythrocytes to group O, Goldstein et al. (Science 215, 168-170, (1982)) used 2000 units of coffee bean α galactosidase per 4.2 ml of packed erythrocytes. This is over 2200 times greater than we have used previously. In order to accomodate experiments at this level it has been necessary for us to concentrate our most highly purified enzyme and to explore conditions and methodology for conversion and assays. The enzyme from hydroxylapatite chromatography was exhaustively dialyzed against water and aliquots were dried in the final reaction tube. The dried enzyme was dissolved in water to remove any residual salts. The purification procedure presently used is described in the following paragraphs.

Purification Procedure

Human placentas stored in a refrigerator from time of delivery were obtained daily from Hutzel Hospital. The membranes, cord and adhering clots were removed under running cold tap water and the placentas were cut into 2" squares and frozen at -16° in sealed plastic bags until use. Storage time varied from 4 to 41 days.

Phenylmethylsulfonyl fluoride (0.5 mM) was prepared by dissolving 1.57 g in 36 ml absolute ethanol at room temperature and adding the mixture to 8 liters of distilled water stirred on a magnetic stirrer at room temperature. After the phenylmethylsulfonyl fluoride was in solution it was moved to the cold room and used only after it had cooled. Two ml of solution were used per gram of placenta. Homogenization was done for 10 min at slow speed in a large capacity Waring blender. The homogenization was done in the cold room (+4 to +6°C) as were all subsequent steps unless mentioned. Usually 2 placentas were homogenized at a time. Each lot was made up of 8 or 9 placentas. In all 12 lots have been fractionated.

The homogenate was strained through 4 thicknesses of cheese cloth suspended in a 30 cm plastic funnel. Three funnels were required. The first 300 ml of filtrate was returned for refiltering since it often contained placental fragments. The process normally took about 40 hr including the time required for 2 half liter washes.

The filtrate was collected in a preweighed plastic bucket and the pooled filtrates weighed. Solid ammonium sulfate (100 g/lb) was gradually added to the stirred filtrate and the stirring was continued for at least 2 hrs after additions. The suspension was allowed to stand for 16-20 hours and then centrifuged at room temperature through a Sharples desk top centrifuge at a flow rate of 2 l/hr or less. The precipitate (36% ammonium sulfate precipitate) was discarded and the supernatant brought to 62% saturation by adding 66.42 g of

ammonium sulfate to each pound of solution. After standing overnight, the suspension was centrifuged as previously and the precipitate was removed from the bowl and weighed. Typical yields were 0.17 g ppt/g placenta. The precipitate was suspended in twice its weight of water and after stirring for about 1 hour was reprecipitated at 62% ammonium sulfate saturation by adding 0.406 g ammonium sulfate/g water. After stirring for an additional hour the precipitate was removed by centrifuging for 1 hour in the Sovall SC2B centrifuge using the G3A rotor at 5000 rpm (4080 G) for 1 hr. The precipitate was suspended in its weight of water and dialyzed against several changes of water over a 60 hour period. The resultant suspension was titrated to pH 5.4 with 0.1 N acetic acid and centrifuged in the Sorvall as previously and washed with one volume of water. The supernatants were stored frozen at -16° until further fractionation.

The DEAE cellulose was regenerated by washing 6 times with 2 volumes of 25% ethanol allowing 20 minutes equilibration, each wash followed by a wash with 95% ethanol. The DEAE was washed twice with 0.1 M pH 6.5 phosphate buffer and then 4 times with 0.01 M pH 6.5 buffer. A 5 x 90 cm column was poured and equilibrated with 0.01 M buffer. In a typical run incoming buffer had a pH of 6.5 and effluent buffer a pH of 6.52. A sample containing 6 g of protein in 800 ml was dialyzed against 3 changes of 0.01 M buffer, centrifuged and applied to the column. Three liters of the buffer was applied to the column and a 3.3 l gradient going from 0 to .25 M NaCl was started followed by a similar linear gradient going from 0.25 M to 0.50 M NaCl. The flow rate initially was 60 ml/hr. The fraction size was 17.5 ml. The fractions containing Azyme activity were combined to form 7 pools. The major peak (2 fractions) accounted for 73% of the recovered material. Total recovery was 59%.

The hydroxylapatite column, 2.5 x 28 cm, was regenerated with 800 ml of 0.20 M sodium phosphate buffer pH 6.5 and equilibrated with 1300 ml of 0.001 M pH 6.5 phosphate buffer. A portion of the major DEAE peak was dialyzed against the 0.001 M buffer and 30 ml containing 582 mg of protein was applied to the column at a flow rate of 24 ml/hr. A 300 ml wash of the buffer followed. A 900 ml linear gradient going from 0.001 to 0.05 M buffer was used to elute the enzyme. The fractions containing Azyme activity were pooled from hydroxylapatite, dialyzed exhaustively against water and lyophilized. Recovery ranged from 50 to 60%. Typical preparations were about 3000 fold purified over the crude homogenate and had specific activities of about 40 $\mu\text{mol hr}^{-1} \text{mg protein}^{-1}$. The peak tube was about 50% more active than the average of the pool. Overall yield is difficult to estimate since appreciable portions of intermediate fractions were used for experiments and assays. Overall recovery is estimated at 20 to 25%.

D. PROPERTIES OF PLACENTAL AZYME

pH Optimum

The activity of the crude placental Azyme was assayed at 10 pH values between 2.31 and 11.13 at 37°C using p-nitrophenyl-2-acetamido-2-deoxy- α -D-galactopyranoside as substrate (Figure 4).

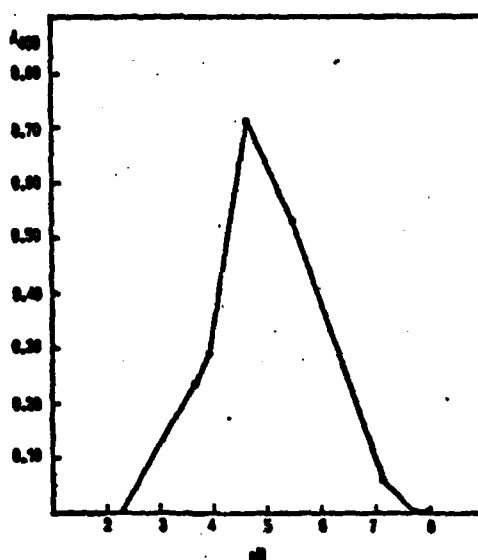


Figure 14. pH optimum of placental Azyme.

The activity was best at pH 4.6 but 76% of the activity remained at pH 5.5 and 8% was left at pH 7.15.

Effect of Temperature on Enzyme Activity

Beutler and Kuhl (J. Biol. Chem. 247, 7195-7200 (1972)) reported that placental Azyme has an unusual temperature dependence. They found that the optimal activity was at 25° using 4 methylumbelliferyl- α -D-galactopyranoside as substrate and that it retained 40% of its activity at 4°. If this were so it would allow one to convert red blood cells at a low temperature. Our preparation of Azyme did not have this property when assayed with p-nitrophenyl-2-acetamido-2-deoxy- α -D-galactopyranoside. At 0° it had 9% as much activity as at 37°.

The assays were done at pH 4.0 in 0.1 M acetate buffer using Azyme which has been purified 58 fold. Each assay was incubated at the indicated temperature for one hour. Four different concentrations of substrate were used and the maximum velocity determined by a Lineweaver-Burk plot.

Temperature	37°	30°	22°	11.5°	0°
Relative velocity	1.0	.65	.53	.33	.09

The reason for the difference between our data and that of Beutler and Kuhl is unknown. Assays were done at different pH's, with different substrates, and with enzymes of different purity.

Heat Stability

The effect of heating on placental Azyme was examined since others have found that similar enzymes are quite stable. This property should be useful for heat inactivation of contaminating enzymes or heat labile viruses. Heating was also studied because some protein impurities might become insolubilized by this treatment. Azyme was quite stable to heat. Its activity was retained or increased during a 2-hour incubation at about 50°C.

Stability studies were done under the following conditions: 50°C in 0.063 M phosphate buffer at pH 6.5, 51°C in 0.10 M acetate buffer at pH 5.4, 51.5°C in 0.10 M acetate buffer pH 4.4 and 60°C in 0.10 M phosphate buffer pH 6.5. All samples which were heated at 50° retained their activity over a 2 hour period. At pH 6.5 the activity at 2 hours was 23% greater than the starting material, at pH 5.4 24% greater and at pH 4.4 1% greater. While there was little precipitate in the samples run at pH 6.5 or 4.4, about 28% of the protein was insolubilized in the run at pH 5.4. The enzyme activity remained in the supernatant. Time studies indicate that in all 3 cases the heated mixture goes through a state where its activity is greater than that of the starting material. At pH 6.5 the maximal increase was 23% at 2 hours. At pH 5.4 it was 59% at 40 min and at pH 4.4 it was 15% at 10 min. The increase (Figure 15) in activity suggested that a thermolabile inhibitor was present. This was further demonstrated by performing assays of mixtures of crude and purified Azyme. The activity of the mixture was less than that of the two fractions measured separately. The purest fraction studied gave a result 76% lower than expected. Heating the enzyme mixtures for 40 min at 50° removed much of the inhibition suggesting that the inhibitor was thermolabile. A stability was also done at 60°. The material was rather labile under these conditions. After 10 minutes 50% of the activity had been destroyed. The end product of the reaction, N-acetylgalactosamine, did not protect the Azyme from inactivation under these conditions. The N-acetylgalactosamine was 14.6 mM. Its K_I is 4.6 mM. The stability to urea and guanidine was also

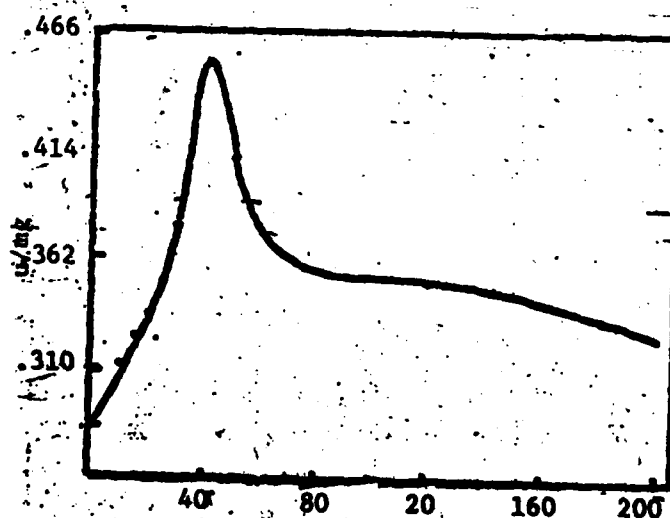


Figure 15. Heat stability of placental Azyme at 50°C.

examined. Ninety % of the activity remained after 1/2 at room temperature in 4M urea but only 33% was left under these conditions when 8 M urea was used. The urea denatured enzyme did not reactivate when the urea was diluted. Azyme incubated at 50° for 30 min in 1 M urea had 79% of its original activity, in 2 M urea 43% was left and in 4 M urea only 5%. Guanidine hydrochloride also denatured the enzyme. After 30 minutes at room temperature 85% was left in 1 M reagent, 37% in 2 M and 2% in 4 M.

Molecular Weight

Placental homogenate was also subjected to gel filtration on a 0.9 x 60 cm column of Sephacryl S 300 (Figure 16).

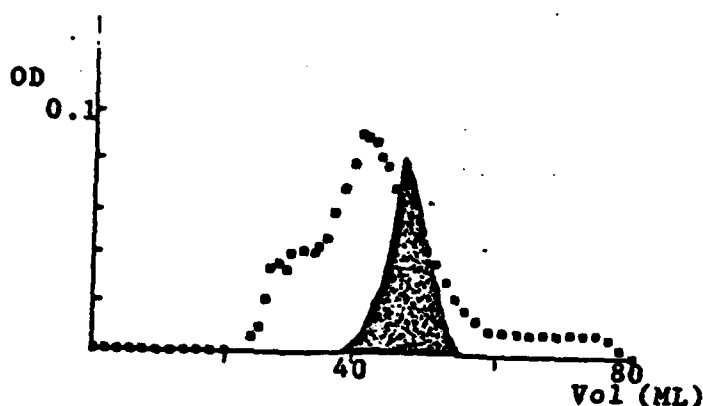


Figure 16. Gel filtration of placental Azyme (shaded area) on Sephacryl S 300.

The enzyme appeared to be single species with a molecular weight of 100,000.

Absence of Sialidase

It is essential that any enzyme preparation used for converting type A cells to type O cells be free of sialidase (neuraminidase) activity since Aminoff et al. ((Proc. Natl. Acad. Sci. 74, 1521-1524 (1977)) have shown that sialidase treated erythrocytes are rapidly eliminated from the circulation and sequestered in the liver and spleen.

The sialidase activity of crude placental extracts and of the partially purified enzyme was determined using fetuin as substrate (Thomas et al., Clin. Genet. 13, 369-379 (1978)). Sialic acid was determined by the procedure of Warren (J. Biol. Chem. 234, 1971-1975 (1959)). Assays were performed at pH 3.5, 4.5, 5.5 and 6.5.

Partially purified *Clostridium perfringens* Azyme was used as a positive control since it is known to contain sialidase. The clostridial enzyme was active at all of the pH's tested. Neither crude nor partially purified placental

Azyme has sialidase activity at pH 6.5 or 5.5. This is encouraging since pH's alkaline to 6.5 will be used for red cell conversion. Crude homogenate had slight activity at pH 4.5 and 3.5. At pH 4.5 it had an activity of .0046 μ moles of sialic acid released per mg protein per hr. At pH 3.5 the activity was .0036. For reference, purified sialidase has an activity of 30 so that even crude extracts contain very little sialidase.

The partially purified enzyme obtained after ammonia sulfate fractionation and precipitation of impurities at low ionic strength was devoid of activity within experimental error yielding less than 3×10^{-4} μ moles of product/mg hr at pH 4.5 and less than 9×10^{-4} μ moles at pH 3.5. Dr. Aminoff also examined a placental Azyme preparation and found it free of sialidase.

The pH profile resembles that of lysosomal sialidase. This enzyme is fairly unstable (Aronson and de Duve, 1968) so that destruction of the enzyme during fractionation and storage may contribute to the absence of activity.

Effect on A Substance

Ammonium sulfate fractionated placental Azyme was tested by David Aminoff at the University of Michigan and found to release hexosamine from type A hog submaxillary glycoprotein. As discussed in the initial section of this report porcine, bovine and human liver enzymes all hydrolyze type A substance.

Isozymes of Azyme

The presence of isozymes of placental Azyme was indicated by the minor additional peaks seen on DEAE and reported previously. These were characterized further by isoelectric focusing. The material precipitating at high ammonium sulfate concentration or eluting at low salt concentrations from DEAE (Figure 17) was enriched in a previously undescribed acidic form which has an isoelectric point near pH 3.6. The major form of the enzyme focuses near pH 4.7. Fractions rich in the isozyme are being collected so that its properties may be delineated and its efficiency in converting A red cells to O determined.

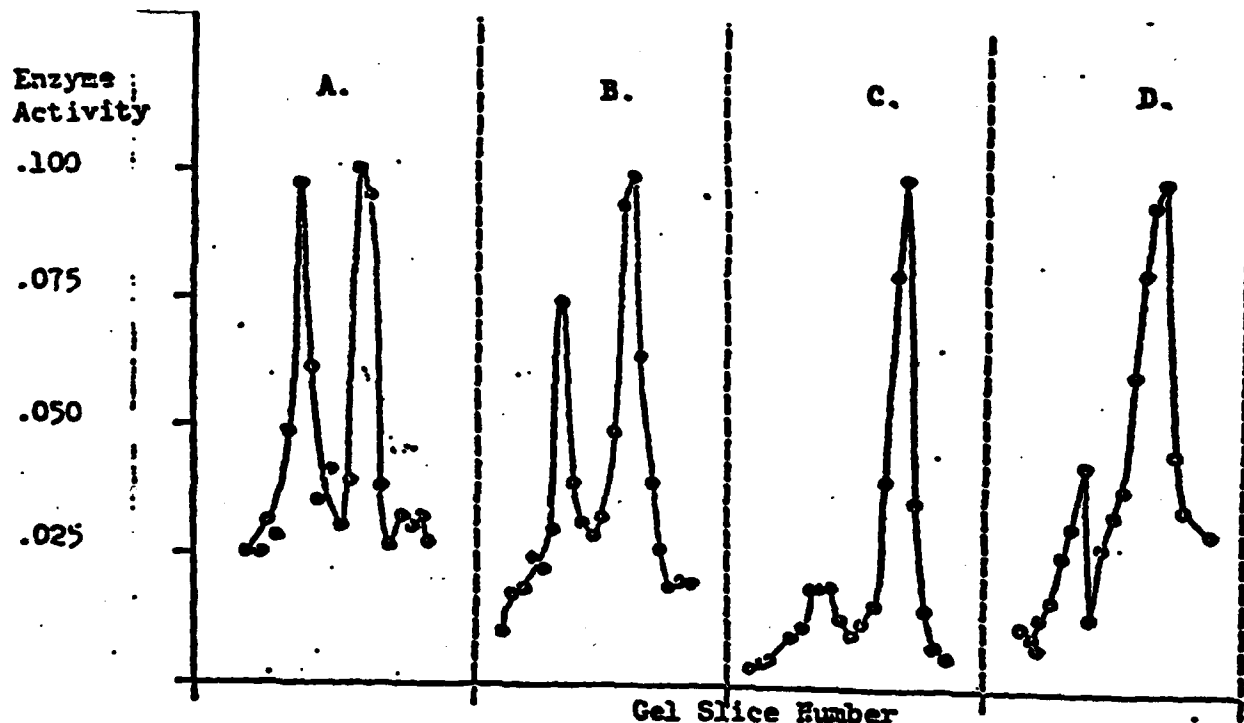


Figure 17. Distribution of Isozymes in Various DEAE Fractions. Two mm slices of isoelectric focusing gels were assayed for Azyme activity. Data for the acidic half of each of 4 gels are shown. Gel A is from a small component preceding the main peak, B, on DEAE chromatography. Gels C and D are from fractions which follow the major DEAE peak.

IV. Assay of Azyme Activity Toward Erythrocytes

In order to monitor the conversion of type A cells to type O a sensitive and quantitative assay is required. It must be sensitive in order to assess completeness of the reaction and quantitative to examine the progress. Four types of agents were studied as primary reactants: typing serum, lectins, competing substrates and monoclonal antibodies. The latter when used in a radioimmunoassay was most successful although the assay still requires additional improvement.

A. ASSAYS USING TYPING SERUM

Two types of assay were investigated using anti A typing serum. In the absorption assay the volume of red cells required to reduce the titer was determined. For a serum active at a 1:512 dilution 10 to 20 μ l of 0.5% cells usually reduced the titer to 1:128. Absorption with type O cells did not reduce the titer. A number of experiments were done using various sera (3 lots), different Azyme preparations, varying incubation times and temperatures and pH's of 5.5, 6.5, 6.8 and 7.2 using various buffers. Although we were very careful the assays had rather poor reproducibility and the results were inconclusive. Difficulty in reading the agglutination near the end point was a major factor causing difficulty.

A binding method was studied next. For this the antibody fraction of the typing serum was partially purified by precipitation with ammonium sulfate at 40% of saturation and labeled with 125 I by the Bolton and Hunter method (Biochem. J. 133, 529-539 (1973)). Only a small number of counts (2% of input) bound to the cells when they were reacted with labeled antibody for 1/2 hr at room temperature. There was little specificity. Another aliquot of the partially purified antibody was further purified by gel filtration of Sephadex G25 and subsequent chromatography on DEAE. It was labeled by the procedure of Banerjee and Ekins (Nature 192, 746-747 (1961)). This serum also lacked specificity. In a typical experiment 250 μ l of dilution of labeled antibody was added to 250 μ l of a 50% cell suspension of type A cells. O cells were used in a control tube. After incubation for 30 min at room temperature the cells were centrifuged and washed twice with saline. The pelleted A cells had 16,900 cpm and the O cells had 18,000 cpm.

The typing serum has a relatively small amount of specific antibody in it. It is pooled from hyperimmunized human donors and contains antibodies to all the minor blood group antigens (Rh, Kell, Duffy, Lewis, Kidd, Lutheran and many others). There appears to be sufficient antibody to these various determinants to mask any specific anti A binding.

B. ASSAYS INVOLVING LECTINS

Binding of lectins labeled with 125 I was also studied as an assay method. Dolichos biflorus (horse gram) lectin which is most widely used for distinguishing type A cells from type O was 125 I labeled by the Bolton-Hunter procedure and separated from unreacted reagent by gel filtration. In experiments where 1.1×10^4 cpm were added to ten fold dilutions of type A and type O red cells starting with 10^8 cells no differences were observed in binding. About 50% of the added counts were bound when 10^8 cells were used. Incubation periods from 1/2 hr to 24 hr did not alter the specificity nor did attempts to absorb

non-specific activity with O cells. Similar results were obtained with red cells from several different donors. (About 9% more counts bound to A cells than O cells.) The procedure (Personal Communication) of Hayes and Goldstein was also used to label the lectin as it has proven useful for lectins which contain sulphhydryl sensitive groups. No differential binding of the labeled lectin to A or O cells could be demonstrated with cells incubated either at 0° or at room temperature for 20 hr. Serial dilutions of A and O cells were also tested against *Dolichos biflorus* lectin but the specificity was not improved. *Bandeiraea simplifolia* lectins BSI & II, the lectins from *Glycine max* (soybean) agglutinin type VI and *Phaseolus limensis* (lima bean) lectin have also been labeled by the method of Hayes and Goldstein. Product protection of the active site of the *Phaseolus* lectin during labeling did not improve specificity. *Bandeiraea* lectin was most promising giving 75% more counts bound to A cells than O.

C. ASSAYS EMPLOYING SUBSTRATE COMPETITION

A series of substrate competition experiments were done. If erythrocyte A substance is a substrate for Azyme and if its concentration is sufficiently high and its K_m small enough, it should compete with p-nitrophenyl- α -N-acetyl-galactosamine. No competition was found. Nor was competition observed with *Clostridium perfringens* enzyme which has been demonstrated to react with A cells. A cells, O cells (as controls), membranes from each and Triton solubilized membranes were used at concentrations up to 100 μ l of packed cells or equivalent per determination. Substrate concentrations between 3 and 30 n molar, pH's of 7.0, 6.7, 4.5, and enzyme activities between .01 and .03 μ mole substrate destroyed per hr were used. All experiments were at 37°C.

The lack of competition suggests that the concentration of A substance is too low since the K_m s for p-nitrophenyl- α -N-acetylgalactosamine (3.3 mM, S.L. Freeman, M.S. Thesis, Wayne State University (1981)) and lipidlike substrates (e.g., Globotriaose, 4.1 mM, Dean and Sweeley, J. Biol. Chem. 254, 9994-10000 (1979)) are similar for human Azyme.

D. ASSAYS WITH MONOCLONAL ANTIBODY TO A SUBSTANCE

Although hemagglutination assays are widely used and very sensitive, they are not very accurate. Usually two fold dilutions are used and most investigators feel that there is a one dilution uncertainty in the results i.e., one cannot determine concentrations within a factor of 2. The monoclonal antibodies were developed for radioimmunoassays which are capable of the greater accuracy required. This is especially important where only small numbers of A determinants remain on the cells. A double antibody procedure was chosen. In it the monoclonal antibodies are reacted with the cells to be tested, unreacted antibody is washed away, and radiolabeled antibody to the monoclonal antibody is added. After the reaction has occurred, the unreacted labeled antibody is removed by washing and the samples are counted. Since the objective of the assay was to initially test the effect of placental Azyme at the concentrations of enzyme used by Goldstein et al. (Science 215, 168-170 (1982)), we have developed a very sensitive assay requiring about 0.0125 μ l of packed red cells. Preliminary work on the assay was done with larger amounts of cells to delineate the proper concentrations of the two antibodies which are used and to optimize the methodologic parameters.

Preparation of monoclonal antibodies to A substance

Eight murine monoclonal antibodies to A substance were prepared in collaboration with Dr. Myron Leon, Professor of Immunology and Microbiology at Wayne. Dr. Leon trained with Dr. Michael Heidelberger and has had extensive experience with antibodies to polysaccharide determinants. The A substance used was a gift from Dr. Elvin Kabat and had been prepared from porcine gastric mucin. Preliminary studies were done with several strains of mice to determine the best responding strain. BALB/c mice gave the highest titers of antibody and were chosen for immunization. Six week old female mice were given 20 µg of A substance in Freund's complete adjuvant subcutaneously. A second injection was given 11 days later in saline. Three days later the spleen was removed and its cells were suspended in RPMI 1640 medium. The erythrocytes were lysed with 0.83% NH_4Cl and the cells were washed twice and fused with P3X 63 6.53 mouse myeloma cells using 30% polyethylene glycol (molecular weight 1000). After 8 min the polyethylene glycol was gradually diluted with medium and the cells were centrifuged and suspended in plating medium, RPMI supplemented with 15% fetal calf serum, glutamine and antibiotics. After overnight culture in a 24 well plate, HAT medium was added. The cells were fed every 2-3 days by removing half of the spent medium and replacing it with fresh HAT medium. After about a week small clones of hybridoma cells were visible. The supernatants were tested against human A and O erythrocytes and the cells which tested positive were expanded on feeder layers of splenocytes on 12 well plates. The cells were cloned by limiting dilution on 96 well plates again using a feeder layer. Dilutions of 50, 20 and 10 viable cells per ml were used. After 5 days the wells were screened for growth and those containing single clones were marked. After 12 days in culture a portion of supernatant was tested. Positive clones were expanded. Aliquots of the expanded clones were frozen in 5% dimethylsulfoxide 95% serum at -70° and the next day stored in a liquid N_2 refrigerator. Ascites tumors were produced in BALB/c mice which had been treated with 0.5 ml of 2, 6, 10, 14 tetramethylpentadecane one week earlier. Peritoneal fluid was collected 2 weeks after immunization.

Preparation of radioiodinated antibodies

Prior to use the reactivity of the goat anti mouse IgM (Meloy Laboratories Batch B203-8) was tested with 3 hybridoma antibodies, 2C6, 2G10 and a monoclonal antibody from Ortho Laboratories. All gave precipitin lines by the Ouchterlony procedure.

The antibody to mouse IgM immunoglobulins was partially purified by precipitation with 50% saturated ammonium sulfate at 4°C . The precipitate was redissolved and reprecipitated with 50% ammonium sulfate. After dialysis the concentration was 30 mg/ml. The goat antibody was labeled by the procedure of Banerjee and Ekins (Nature 192, 746-747 (1961)) using 36 mg of protein and 2 mCi of Na^{125}I . After 2 hr at room temperature reaction was stopped and the labeled antibody was separated from $^{125}\text{I}^-$ by gel filtration on a 22.5 x 1.5 cm column of G25 medium Sephadex. Good separation was achieved. The labeled antibody contained 2.8×10^4 cpm/µg protein (32 dpm/nmole). Later in the experiments a similar lot was prepared. For the experiments with very small amounts of cells a more heavily labeled batch of the antibody was prepared by labeling only 6 mg of protein. The protein concentration of that labeled material was not determined but the specific activity is estimated to be about 7.5×10^5 cpm/µg (840 dpm/nmole). A single batch of hybridoma

protein was also labeled to compare the sensitivity of a single antibody technic with the double antibody procedure and to determine if labeling both antibodies increased the sensitivity. The labeled antibodies were stored frozen in 1% HSA containing 0.02% sodium azide. The reactivity of the radiolabeled antibodies decreased more rapidly than expected from the half life of ^{125}I over 1 or 2 months of storage. This was probably due to radiation damage. Recently the Hayes and Goldstine procedure for labeling has been used. Antibodies labeled by this technic appear to have higher specificity.

Studies of reaction conditions using larger amounts of cells

Preliminary studies to optimize the assay were done with 0.25 ml of 2% A and 0 cells (i.e., 0.5 μl of packed cells). For most of the experiments the cells were from the same donors and were used for no longer than two weeks. The number of cells was estimated according to the procedure of Kabat (Experimental Immunochemistry 2nd Ed. p. 150). We plan to use a Coulter counter in future experiments.

Choice of first antibody

Three hybridoma antibodies 2C6, 2G10 and the Ortho antibody were compared to determine which gave the best results in the double antibody system. All were tested by hemagglutination and the last dilution giving + hemagglutination was used for the radioimmunoassay. All gave comparable results and 2C6 was chosen for further study because a large amount was available.

Dilution of first antibody

The objective of the assay is to saturate the red cells with first (hybridoma) antibody. The 2C6 antiserum was absorbed with 0 cells to remove any non-specific antibody. (The 2C6 was from mouse ascites fluid and might have contained Forasman or similar antigens). Absorption did not change the non-specific counts. Table I summarizes experiments with the hybridoma. A dilution of 1/256 was used in most subsequent experiments. The large values for the control tube are due to non-specific attachment of the labeled anti IgM. Reduction in these counts required much work which will be outlined in the following pages.

Experiments to diminish background counts

A large number of experiments were done to diminish the background counts. Most were non-specifically absorbed to the tubes used. Addition of 1% bovine serum albumin (BSA) decreased the background about 11 fold. Use of 4% BSA decreased the background to about 1/2 that with 1% BSA. Glass and polyethylene tubes gave about the same background. Siliconizing did not improve the background. Since most of the background counts are on the centrifuge tube walls, it would be expected that transferring the labeled cells to a fresh tube would help. Since some of the cells remained in the pipette tip it was necessary to count it with the transferred cells.

Variables affecting accuracy

The hybridoma antibody tends to clump the A erythrocytes but not the O cells. Several methods of resuspending them without causing lysis were studied. Pipetting with a plastic tipped pipet was inferior to gentle mixing on a Vortex mixer. Resuspending the cells every 10 minutes during the reaction improved the results marginally.

Proportionality to number of red cells

The range over which the assay is operative was determined to be between 2.5×10^7 and 2.5×10^8 cells using the system with a volume of 0.4 ml (Figure 18). This system was also tested with mixtures of A and O cells. A good proportionality was observed between the proportion of A cells and the radioactivity (Figure 19).

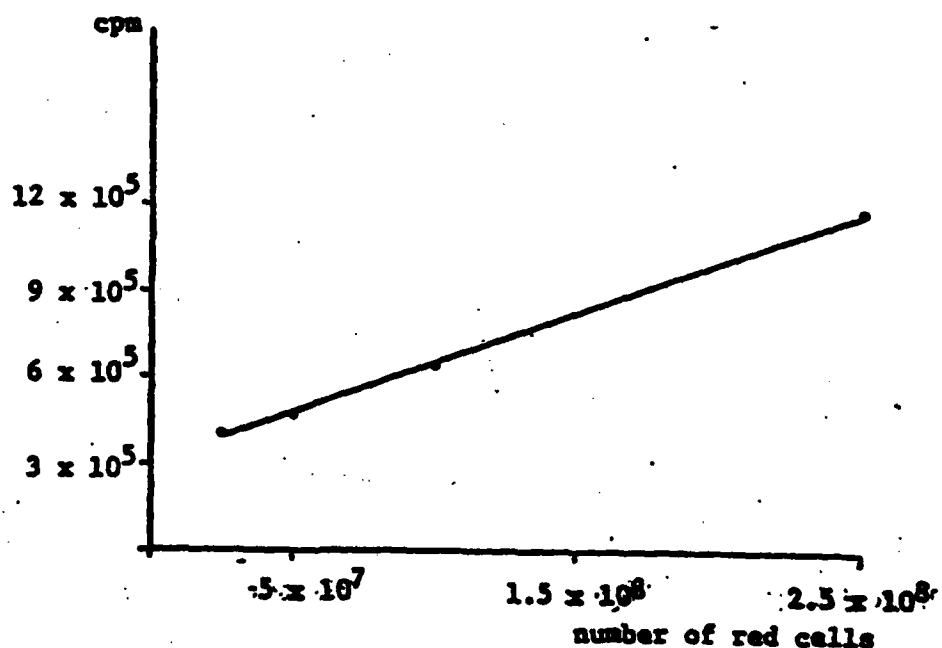


Figure 18. Proportionality between number of A cells and radioactivity.

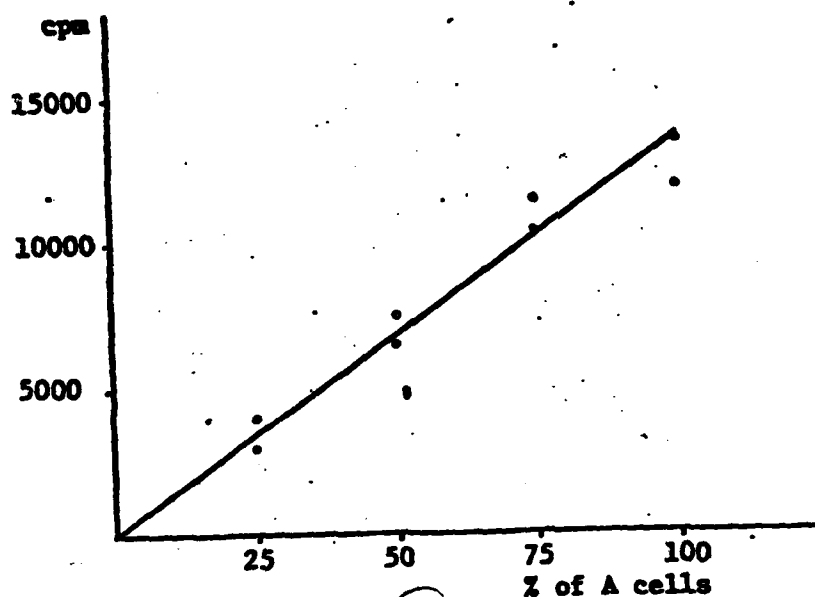


Figure 19. Proportionality with content of A cells

Comparison of single antibody and double labeled antibody systems

The hybridoma antibody was labeled with radioactive iodine and dilutions were tested with a constant amount of labeled hybridoma antibody. There was less specificity in the single label assay. In all experiments antibody and cells were reacted at 37° for 1 hr. One set was allowed to stand at 4° for 20 hr while the other was assayed at once. It was better to assay at once. A double antibody system was also compared. In it both the first and second antibodies were ¹²⁵I labeled. Using labeled first antibody only A and O cells gave a difference of 10,000 cpm, with both antibodies labeled 17,700 cpm and with labeled second antibody 23,000 cpm.

Development of a micro assay

There is agglutination of A cells and not O cells during the assay. Resuspension of the A cells occasionally causes some lysis. Two methods of ensuring that lysed cell membranes would be retained were examined. Cell membranes were quantitatively retained on 0.22 μ m Millipore membranes. Pretreatment of the membranes with 4% BSA was necessary to avoid non-specific interactions. Stroma tended to dissociate from the filter during the incubations with antibody. No satisfactory way overcoming this was found. Centrifugation at 20,000 G was also studied and was the preferred method. The optimal concentrations of first antibody (a 1/128 dilution of 2C6) and second antibody (1/2) were determined as previously. The proportionality

with number of cells was determined (Figure 20). The blanks were lower than in the experiments at larger volume since only a small portion of the tube is in contact with the reaction mixture.

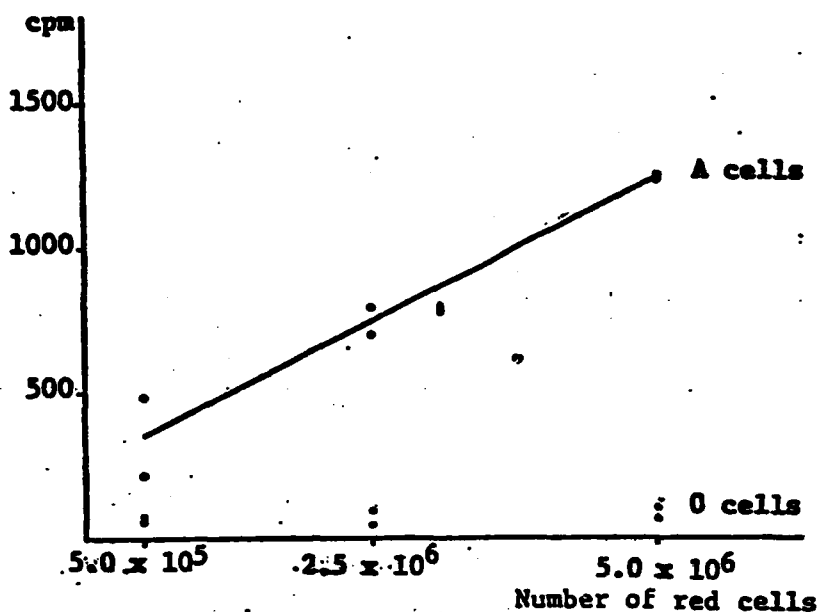


Figure 20. Proportionality between number of cells and radioactivity in micro assay.

The procedure presently in use is as follows.

Red cells are washed 3 times with phosphate buffered saline (PBS) pH 7.2 and a 5% suspension is made. Its concentration is determined by adding 0.1 ml of suspension to 1.4 ml of 0.1% Na_2CO_3 , reading the absorbance at 541 nm and adjusting the volume of the suspension until it reads .700. A 1.25% cell suspension is made by diluting with 3 volumes of PBS containing 4% BSA. Ten μl of this suspension is placed in a 0.5 ml polypropylene microfuge tube (Sarstedt 72.699) which has been soaked for 24 hr in 4% BSA in PBS at 4°C. The test is done in duplicate with a pair of tubes containing 0 cells serving as controls. Tubes without cells may also be run. Next 10 μl of a 1:1965 dilution in PBS-BSA of first antibody from hybridoma 2C6 is added and mixed with the cells on a Vortex mixer. The mixture is incubated for 15 min at 37° with the tubes being mixed again half way through the incubation. The tubes are next centrifuged for 1/2 hr at 20,000 G in a Sorvall refrigerated centrifuge using an SS 34 rotor at 13,000 rpm. After a 1/2 hr centrifugation the supernatants are carefully removed with a syringe and the pellets are covered with 100 μl of PBS-BSA disturbing them as little as possible. The centrifugation is repeated and the supernatant is again carefully removed. Ten μl of a 1:2 dilution of the ^{125}I labeled second antibody is added carefully so that the pellet is not disturbed and the reaction is incubated at 37° for 15 min. After centrifugation the tube is counted to determine the input counts. (The blanks are quite sensitive to variation in the input counts.) The supernatants were then removed and the pellets were washed 3 times, transferred to fresh tubes using four 50 μl aliquots of PBS-BSA, and counted.

Assays performed in duplicate on two successive days and in triplicate on the third gave 218, 219 and 194 cpm differences between the A cell and 0 cell values.

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